D-Amino Acid Oxidase Activator (DAOA/G72) Pathways and its Role in Schizophrenia

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1. Summary

Schizophrenia is a complex heritable psychiatric disorder, which is characterised by positive and negative symptoms, and cognitive deficits. This thesis focusses on the glutamate hypothesis of schizophrenia, which is based on the hypofunction of N-methyl-D-aspartate (NMDA) receptors. This hypothesis is thought to explain the negative symptoms and cognitive deficits better than the dopamine hypothesis based on the observed beneficial effect of NMDA receptor modulators on these symptoms. NMDA receptors require both glutamate and co-agonist such as glycine or D-serine to function normally. D-serine is degraded in the brain by the D-amino acid oxidase (DAO) enzyme. Thus, DAO can regulate the function of NMDA receptors via D-serine breakdown. One molecular explanation proposed for the NMDA receptor hypofunction is increased DAO activity leading to decreased D-serine which ultimately results in NMDA receptor hypofunction. The cause of increased DAO activity in schizophrenia has been proposed to be due to its interaction with the DAO activator (DAOA/G72). DAOA has been reported to both increase and decrease DAO activity. Thus, the effect of DAOA on DAO activity is still controversial. Despite 15 years of research in the field of DAOA, the exact function of DAOA, its expression pattern in the human brain and its interaction with DAO is yet to be elucidated.

The main objectives of this thesis were (i) to understand DAO and DAOA mRNA and protein expression pattern trajectories across six brain regions during development and aging in the human post-mortem brains, (ii) to re-evaluate association of *DAO* and *DAOA* single nucleotide polymorphisms (SNPs) with schizophrenia by conducting meta-analysis of case-control schizophrenia association studies, (iii) to assess *DAO* and *DAOA* SNPs as predictive genetic and endophenotype markers for transition to schizophrenia-spectrum disorders in at-risk for psychosis individuals, and (iv) to determine the effect of DAOA on DAO and NMDA receptor activity in human cell lines.

In the post-mortem study (study I), human post-mortem samples from six brain regions (ages: 16 weeks of gestation to 91 years) with no neuropathological evidence of neuropsychiatric disorders were used. We found a higher *DAO* mRNA levels in the cerebellum, whereas lower DAO protein levels in the cerebellum compared to other brain regions studied (brainstem, amygdala, striatum, thalamus, and frontal cortex), which might suggest post-transcriptional regulation. Furthermore, DAOA protein was detectable in all brain regions

studied, whereas *DAOA* mRNA was undetectable in these brain regions, which might suggest a tightly regulated or extremely localised DAOA expression. We found a positive correlation between DAO and DAOA proteins in all brain regions studied except in the frontal cortex, which provides evidence for simultaneous expression of these proteins. Moreover, *in silico* DNA methylation data downloaded from Gene Expression Omnibus (GEO) datasets showed that *DAO* and *DAOA* CpG sites in the cerebellum were significantly more methylated than in the frontal cortex, which might explain the brain-region specific differential DAO and DAOA expression.

In the meta-analysis study (study II), we performed a systematic literature search which resulted in 20 *DAO* and 23 *DAOA* case-control schizophrenia association studies that fulfilled the inclusion criteria. We re-evaluated the association of 8 *DAO* and 12 *DAOA* SNPs with schizophrenia. Out of these SNPs, only one *DAO* (rs4623951), and two *DAOA* (rs778293 and rs3916971) SNPs were significantly associated with schizophrenia. This systematic meta-analysis is the most updated one for the association of *DAO* and *DAOA* SNPs with schizophrenia.

The at-risk for psychosis study (study III) included a 3-year prospective study cohort of 185 individuals (ages: 13-35 years) at clinical risk for psychosis, who were followed-up at 6, 12, 24, and 36 months for conversion to schizophrenia-spectrum disorders. We assessed whether *DAO* (rs3918347, rs4623951) and *DAOA* (rs778293, rs3916971, rs746187) SNPs could predict conversion as well as whether they show association with Research Domain Criteria (RDoC, a non-disease-based dimensional approach) domains, namely, negative valence system (anxiety, hopelessness) and cognitive systems (perception disturbances, disorganised symptoms). We found that *DAOA* rs746187 CC versus CT+TT genotype carriers (T-allele: risk for schizophrenia) and *DAOA* rs3916971 TT versus TC+CC genotype carriers (T-allele: protective against schizophrenia) experienced nominally more hopelessness and more visual perception disturbances, respectively. Furthermore, *DAO* rs3918347 GA+AA genotype carriers experienced nominally more auditory perception disturbances than GG genotype carriers (G-allele: risk for schizophrenia). Moreover, *DAO* and *DAOA* SNPs did not emerge as predictive markers for conversion to schizophrenia-spectrum disorders at 36 months follow-up in at-risk for psychosis population. We found that the schizophrenia risk G-allele of *DAO* rs3918347 nominally increased the risk for ultra-high risk (UHR) for psychosis individuals with attenuated positive symptoms syndrome (APSS). Our results suggest that *DAO* and *DAOA* SNPs might be predictive markers for endophenotypes such as RDoC negative valence and cognitive systems in at-risk

for psychosis population.

In the cell culture study (study IV), we used human neuroblastoma SH-SY5Y, human astrocytoma 1321N1, and human embryonic kidney HEK293 cells. DAO activity was measured based on the release of hydrogen peroxide and its interaction with Amplex Red reagent in all three cell lines co-transfected with DAO and DAOA plasmid constructs. NMDA receptor activity was measured using whole-cell patch-clamp recording in HEK293 cells that were stably transfected with NMDA receptor subunits NR1 and NR2A (NR1/NR2A HEK293) and transiently transfected with DAOA plasmid construct. *In silico* experiments were performed using Molecular Dynamics simulations. We found that DAOA increased DAO activity only in human kidney-like HEK293 cells, but DAOA had no effect on DAO activity in human neuron-like SH-SY5Y and human astrocyte-like 1321N1 cells. This might be because of different signalling pathways, different compartmentalization of DAO and DAOA proteins and lower DAO and DAOA overexpressed protein levels in 1321N1 and SH-SY5Y cells compared to HEK293 cells. Furthermore, DAOA had no effect on NMDA receptor activity in NR1/NR2A HEK293 cells. The simulation experiments showed that DAOA makes human DAO (hDAO) holoenzyme [hDAO with flavin adenine dinucleotide (FAD)] more flexible and misfolded than hDAO apoprotein (hDAO without FAD), that might be suggestive of inactivating effect of DAOA on hDAO, which is not consistent with our DAO activity results. Thus, the interactions between DAO and DAOA still needs to be elucidated in a more realistic *in vitro* cellular model that recapitulates the complex signalling of tripartite synapse.

In summary, the performed studies showed that *DAO* and *DAOA* genes are highly regulated at the level of transcription, and have brain-specific expression patterns varying with age, which might suggest that these genes are important for brain development. Furthermore, *DAO* and *DAOA* SNPs might emerge as predictive markers for endophenotypes such as negative symptoms and cognitive deficits in at-risk for psychosis population. Moreover, the role of DAOA in the NMDA receptor hypofunction pathway is still obscure and needs to be elucidated with the hope that DAOA might emerge as a therapeutic target for the treatment of negative symptoms and cognitive deficits in schizophrenia.

2. Zusammenfassung

Schizophrene Störungen sind komplexe psychiatrische Erkrankungen, die meistens in der Adoleszenz oder im jungen Erwachsenenalter beginnen, zum grossen Teil genetisch bedingt sind und durch sogenannte "positive" (z.B. produktive Symptome wie Halluzinationen oder Wahn) und "negative" Symptome (z.B. Defizite und Reduktion im Antrieb und Affekt), sowie kognitive Defizite charakterisiert sind. Die vorliegende Dissertation konzentriert sich auf die Glutamathypothese der Schizophrenie, die auf einer Hypofunktion der N-Methyl-D-Aspartat-(NMDA)-Rezeptoren basiert. Es wird vermutet, dass die Dysfunktionen im glutamatergen System die Entwicklung von "negativen Symptomen" und "kognitiven Defiziten" besser erklärt als die sogenannte Dopaminhypothese. Diese Annahme basiert u.a. auf den beobachteten positiven Auswirkungen von NMDA-Rezeptor-Modulatoren auf die erwähnten Symptome. NMDA-Rezeptoren benötigen sowohl Glutamat als auch Co-Agonisten wie Glycin oder D-Serin für eine normale Funktion. Im Gehirn wird D-Serin durch das Enzym D-Aminosäureoxidase (DAO) degradiert. Folglich kann DAO die Funktion des NMDA-Rezeptors durch den Abbau von D-Serin regulieren. Eine mögliche molekulare Erklärung für die Hypofunktion des NMDA-Rezeptors ist eine gesteigerte DAO-Aktivität, die zu reduzierter D-Serin-Aktivität führt, was schliesslich eine NMDA-Rezeptor-Hypofunktion generieren kann. Die Ursache für die erhöhte DAO-Aktivität bei schizophrenen Störungen wird in der Interaktion von DAO mit dem DAO-Aktivator (DAOA/G72) vermutet. Es wurde beschrieben, dass DAOA die DAO-Aktivität sowohl steigern als auch vermindern kann. Bis heute wird der Effekt von DAOA auf die DAO-Aktivität noch immer kontrovers diskutiert. Obwohl DAOA schon seit vielen Jahren Gegenstand der Forschung ist, ist seine exakte Funktion, das Expressionsmuster im menschlichen Gehirn und die Interaktion mit DAO weiterhin ungeklärt.

Ziel dieser Dissertation war einerseits Verläufe und Beziehungen von *DAO*- und *DAOA*-mRNA- sowie DAO- und DAOA-Protein-Expressionsmuster in sechs verschiedenen Hirnregionen im Verlaufe der Entwicklung und des Alterungsprozesses im menschlichen Gehirn mittels post-mortem Gehirnproben zu verstehen. Andererseits sollte der Zusammenhang von *DAO*- und *DAOA*-Einzelnukleotid-Polymorphismen (SNPs, engl. single nucleotide polymorphism) und schizophrenen Störungen mittels einer Metaanalyse von Fall-Kontroll-Assoziationsstudien neu bewertet werden. Dies um *DAO*- und

DAOA-SNPs als prädiktive genetische und endophänotypische Marker für die Transition von Risikopatienten zu einer Schizophrenie-Spektrumsstörung zu bestimmen. Ein weiteres Ziel war es, den Effekt von DAOA auf DAO und die NMDA-Rezeptor-Aktivität in menschlichen Zelllinien zu ermitteln.

Für die erste Fragestellung (Studie I) wurden post-mortem Proben aus sechs verschiedenen Hirnregionen (Alter: 16. Schwangerschaftswoche bis 91 Jahre) verwendet, bei denen keine neuropathologischen Hinweise für eine neuropsychiatrische Erkrankung vorhanden waren. Wir fanden höhere *DAO*-mRNA-Levels und gleichzeitig tiefere DAO-Protein-Levels im Cerebellum, verglichen mit den anderen untersuchten Hirnregionen (Hirnstamm, Amygdala, Striatum, Thalamus und frontaler Kortex), was möglicherweise auf eine post-transkriptionale Regulation hindeutet. Ausserdem war das DAOA-Protein in allen untersuchten Hirnregionen nachweisbar. *DAOA*-mRNA hingegen konnte in diesen Regionen nicht nachgewiesen werden, was auf eine streng regulierte oder extrem lokalisierte DAOA-Expression hindeuten könnte. Die DAO- und DAOA-Proteine zeigte in allen untersuchten Hirnregionen ausser im frontalen Kortex eine positive Korrelation, was auf eine gleichzeitige Expression dieser beiden Proteine hinweist. Ausserdem haben *in silico* DNA-Methylierungsdaten, die vom Gene-Expression-Omnibus-(GEO)-Datensatz heruntergeladen wurden, gezeigt, dass *DAO*- und *DAOA*-CpG-Motive im Cerebellum signifikant stärker methyliert sind als im frontalen Kortex. Dies erklärt möglicherweise die regionenspezifisch unterschiedliche DAO- und DAOA-Expression.

Für die Metaanalyse (Studie II) erfolgte eine systematische Literaturrecherche, die 20 *DAO*- und 23 *DAOA*-Fall-Kontroll-Schizophrenie-Assoziationsstudien ergab, welche die Einschlusskriterien erfüllten. Es wurden Assoziationen von 8 *DAO*- und 12 *DAOA*-SNPs mit Schizophrenie beurteilt. Von diesen SNPs waren ein *DAO*- (rs4623951) und zwei *DAOA*-SNPs (rs778293 und rs3916971) signifikant mit schizophrenen Störungen assoziiert. Diese systematische Metaanalyse ist die aktuellste Studie zur Assoziation von *DAO*- und *DAOA*-SNPs mit schizophrenen Störungen.

Die klinische Studie (Studie III) beinhaltete eine dreijährige prospektive Kohortenstudie von 185 eingeschlossen Individuen (Alter: 13-35 Jahre) mit einem klinischen Risiko für schizophrene Störungen, die nach 6, 12, 24 und 36 Monaten auf Anzeichen und Symptome für eine Schizophrenie-Spektrumsstörung untersucht wurden. Ziel war die Untersuchung, ob *DAO*-SNPs (rs3918347, rs4623951) und *DAOA*-SNPs (rs778293, rs3916971, rs746187) die Entwicklung

der Erkrankung vorhersagen können und ob sie eine Assoziation mit folgenden Research-Domain-Criteria-(RDoC)-Domänen (einem nicht-krankheitsbasierten, dimensionalen Ansatz) "negatives Valenzsystem" (Angst, Hoffnungslosigkeit) und "kognitive Systeme" (Wahrnehmungsstörungen, desorganisierte Symptome) zeigen. Wir stellten fest, dass *DAO* rs746187 CC-Allelträger versus CT- und TT-Allelträger (z.B. T-Allel: erhöhtes Risiko für schizophrene Störungen) und *DAOA* rs3916971 TT-Allelträger versus TC- und CC-Allelträger) eine nominal stärkere Hoffnungslosigkeit und visuelle Wahrnehmungsstörung erlebten. Ausserdem erfuhren *DAO* rs3918347 GA- und AA-Allelsträger nominal häufiger an auditiven Wahrnehmungsstörungen als GG-Merkmalsträger. Darüberhinaus stellten sich die *DAO*- und *DAOA*-SNPs nicht als prädiktive Marker für die Entwicklung einer Schizophrenie-Spektrumsstörung in der Risikopopulation nach 36 Monaten heraus. Wir stellten jedoch fest, dass das Schizophrenierisiko-G-Allel von *DAO* rs3918347 das Risiko für ultra-high risk (UHR) bei Psychosepatienten mit attenuated positive symptoms syndrome (APSS) nominal erhöht. Unsere Ergebnisse weisen möglicherweise darauf hin, dass *DAO*- und *DAOA*-SNPs zwar keine generellen prädiktiven Marker für die Entwicklung von schizophrenen Störungen sind, aber prädiktive Marker für gewisse Endophänotypen, beispielsweise für RDoC-Domänen des negativen Valenzsystems und des kognitiven Systems bei Psychose-Risikopopulationen darstellen könnten.

In der Zellkulturstudie (Studie IV), wurden menschliche SH-SY5Y Neuroblastoma-Zellen, menschliche 1321N1 Astrozytom-Zellen, und menschliche HEK293 embryonale Nierenzellen verwendet. Die DAO-Aktivität wurde mittels freigegebenen Wasserstoffperoxids und dessen Interaktion mit dem Amplex-Red-Reagenz in allen drei Zelllinien, die mit DAO- und DAOA-Plasmidkonstrukten co-transfiziert wurden, gemessen. Die NMDA-Rezeptoraktivität wurde mit dem whole-cell-patch-clamp-Verfahren in HEK293-Zellen gemessen, die mit den NMDA-Rezeptor-Untereinheiten NR1 und NR2A (NR1/NR2A HEK293) stabil transfiziert und mit dem DAOA-Plasmidkonstrukt transient transfiziert wurden. *In silico* Experimente wurden mit Molekulardynamik-Simulationen durchgeführt. Wir stellten fest, dass DAOA die DAO-Aktivität nur in humanen embryonalen HEK293-Nierenzellen erhöht war, nicht aber in humanen neuronalen SH-SY5Y-Zellen und humanen 1321N-Astrozytenzellen. Dies könnte möglicherweise aufgrund verschiedener Signalwege, unterschiedlicher Kompartimentierung von DAO- und DAOA-Proteinen, sowie tieferer DAO- und DAOA-Proteinüberexpression in 1321N1-Zellen und SH-SY5Y-Zellen

im Vergleich zu HEK293-Zellen bedingt sein. Des Weiteren hatte DAOA keinen Effekt auf die NMDA-Rezeptor-Aktivität in NR1/NR2A HEK293-Zellen. Die Simulationsexperimente zeigten, dass DAOA das humane DAO-(hDAO)-Holoenzym [hDAO mit Flavin-Adenin-Dinukleotid (FAD)] flexibler zu machen scheint und auch eine stärkere Dysfunktion in der Faltung verursachen könnte als das hDAO-Apoprotein (hDAO ohne FAD), was auf einen inaktivierenden Effekt von DAOA auf hDAO hinweist. Der letztgenannte Befund erscheint jedoch nicht konsistent mit unseren Resultaten zur DAO-Aktivität. Daher sollte die Interaktion zwischen DAO und DAOA weiter untersucht werden z.B. mittels eines noch geeigneteren "*in vitro* Zellmodells", das die komplexe Zellkommunikation von tripartiten Synapsen realistisch abbildet.

Zusammengefasst zeigen die vier durchgeführten Studien, dass die *DAO*- und *DAOA*-Gene auf transkriptioneller Ebene stark reguliert sind und hirnspezifische Expressionsmuster aufweisen, die altersabhängig variieren, was darauf hindeutet, dass diese Gene wichtig für die Gehirnentwicklung sind. *DAO*- und *DAOA*-SNPs als prädiktive Marker für Endophänotypen, wie negative Symptome und kognitive Defizite in Psychoserisiko-Populationen sollten repliziert werden. Aufgrund der bisherigen und hier vorgelegten Studien erscheint die Rolle von DAOA für die Entwicklung einer NMDA Rezeptorhypofunktion noch immer unklar und sollte weiter erforscht werden, in der Hoffnung, dass DAOA möglicherweise als ein therapeutisches Target zur Behandlung von negativen Symptomen und kognitiven Defiziten bei schizophrenen Störungen dienen kann.

3. General Introduction

3.1 Schizophrenia: symptomatology, diagnostic criteria, and epidemiology

Schizophrenia is a severe, chronic, and debilitating psychiatric disorder. It is characterised by positive symptoms such as delusions, hallucinations, and thought disorder, and negative symptoms such as affective flattening or blunting, avolition, apathy, anhedonia, alogia, and asociality (Crow, 1985; Andreasen, 1995; van Os and Kapur, 2009; Stahl, 2013). Although cognitive deficits such as impairments in attention, memory, and executive functions are not formally recognised as part of the diagnostic criteria for schizophrenia, numerous studies use this symptom subcategory in schizophrenia research (Elvevag and Goldberg, 2000; Fioravanti et al., 2012; Stahl, 2013).

The diagnosis of schizophrenia still relies on the examination of the mental state through a clinical interview and observation of patient's behaviour because of the absence of biological markers for schizophrenia. The diagnostic guidelines for schizophrenia are provided by two major classification systems, namely Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (American Psychiatric Association, 2013) and 10^{th} revision of the International Classification of Diseases (ICD-10) (World Health Organization, 1992). According to DSM-5 criteria, Schizophrenia Spectrum and Other Psychotic Disorders include schizophrenia (295.90), other psychotic disorders (298.80 and 298.90), and schizotypal personality disorder (295.40) (American Psychiatric Association, 2013). In ICD-10 criteria, schizophrenia (F20) includes paranoid schizophrenia (F20.0), hebephrenic schizophrenia (F20.1), catatonic schizophrenia (F20.2), undifferentiated schizophrenia (F20.3), post-schizophrenic depression (F20.4), residual schizophrenia (F20.5), simple schizophrenia (F20.6), other schizophrenia (F20.8), and unspecified schizophrenia (F20.9) (World Health Organization, 1992). There are some major differences between ICD-10 and DSM-5 criteria for schizophrenia. ICD-10 criteria puts greater emphasis on Schneiderian first-rank symptoms (Schneider, 1959), classic schizophrenia subtypes, non-dimensional assessment of schizophrenia, and 1-month duration for the diagnosis of schizophrenia. However, DSM-5 criteria eliminated special emphasis on Schneiderian first-rank symptoms, deleted classic schizophrenia subtypes, added a dimensional assessment to characterise the heterogeneity of schizophrenia, and 6-month duration for the diagnosis of schizophrenia (Barch

et al., 2013; Tandon, 2014).

Schizophrenia is a serious public health problem, which affects more than 21 million people worldwide (World Health Organisation, 2016). The incidence of schizophrenia varies from 0.1 to 0.4 per 1000 population (Jablensky et al., 1992). The lifetime prevalence of this disorder is estimated to be 0.3-0.7% (McGrath et al., 2008). Schizophrenia is a major burden for the healthcare systems worldwide, which accounts for 1.5-3% healthcare expenses in developed countries (Knapp et al., 2004). The economic burden of schizophrenia in the United States of America (USA) in the year 2013 was estimated to be between $134.4 billion and $174.3 billion, which included direct health costs (24%), direct non-health care costs (6%), and indirect costs (75%) (Cloutier et al., 2016). Thus, the economic burden of the disorder highlights the need for effective pharmacological interventions to increase the stability and performance of schizophrenia patients which in turn would help in addressing the healthcare costs.

3.2 Treatment of schizophrenia

The current treatment of schizophrenia involves both pharmacotherapy and psychotherapy (Huhn et al., 2014). The antipsychotic medications are the first-line of pharmacotherapy in schizophrenia (Patel et al., 2014). Antipsychotics are classified into two groups, namely first-generation antipsychotics (typical antipsychotics) that emerged in the 1950s and second-generation antipsychotics (atypical antipsychotics) that emerged in the 1980s (Leucht et al., 2009). First-generation antipsychotics (e.g., chlorpromazine, haloperidol) are dopamine D2 receptor antagonists, which are effective in reducing positive symptoms of schizophrenia. However, these first-generation antipsychotics are ineffective and may exacerbate negative symptoms and cognitive impairment of schizophrenia, and also cause adverse effects such as extrapyramidal symptoms (tremor, rigidity, dystonia, bradykinesia), hyperprolactinemia, and cognitive dulling (Li et al., 2016). Second-generation antipsychotics (e.g., clozapine, risperidone, aripiprazole) are a combination of dopamine D2 receptor and serotonin 5-HT2A receptor antagonists that are effective against positive symptoms, but less effective against negative symptoms and cognitive impairment in schizophrenia. These second-generation antipsychotics have reduced the propensity to cause extrapyramidal symptoms and hyperprolactinemia than first-generation antipsychotics, however, they can still cause adverse effects such as weight gain and metabolic disorders (Divac et al., 2014). Many schizophrenia patients (around 10-30%) fail to respond to antipsychotic treatment, some patients are non-compliant due to adverse effects, and antipsychotic responders are still

incapable of living a normal productive life due to persistence of negative symptoms and cognitive impairment (Fenton et al., 1997; Galuppi et al., 2010; Hasan et al., 2012). Thus, there is an urgent need to develop new drugs for the treatment of negative symptoms and cognitive impairment in schizophrenia.

3.3 Aetiology of schizophrenia

3.3.1 Genetic factors

Schizophrenia is a complex heritable disorder with non-Mendelian inheritance. The heritability of this disorder is around 60-80% (Pepper and Cardno, 2014). The best known genetic risk factors for schizophrenia are single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) (Harrison, 2015). Genome-wide association studies (GWAS) have provided unequivocal evidence for common SNPs contributing to schizophrenia risk. A study by the Psychiatric Genetics Consortium (PGC) involving 21,000 cases and 38,000 controls identified 22 loci, which contain SNPs with genome-wide significance for schizophrenia (Ripke et al., 2013). This study estimated that over 8,000 SNPs independently contribute to schizophrenia, and which together will explain over 50% of the genetic predisposition (Ripke et al., 2013). A new analysis from the PGC involving 37,000 cases and 113,000 controls identified over 100 loci (implicating about 600 genes) with genome-wide significance for schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Thus, schizophrenia is a highly polygenic disorder (Lee et al., 2012). Most of the schizophrenia-associated SNPs are in the non-coding regions of DNA (e.g., intergenic or intronic), which makes identifying the biological basis for these SNPs very challenging (Harrison, 2015). Each schizophrenia-risk SNP was found to have a very small effect size on schizophrenia risk (Harrison, 2015). In contrast to schizophrenia-associated SNPs, each CNV was found to be extremely rare but penetrant, which confers significantly increased risk for the disorder (Kirov et al., 2014). Many CNVs associated with schizophrenia occur *de novo* (Xu et al., 2011) and the others are inherited. The extent to which CNVs contribute to the overall aetiology of schizophrenia is unknown. Nowadays, there is increasing empirical and bioinformatic evidence of schizophrenia implicated genes converging upon biochemical pathways and networks (Harrison, 2015).

3.3.2 Environmental factors

The aetiology of schizophrenia cannot be explained completely by genetic risk factors, which are highlighted by the fact that concordance rates in monozygotic twins are around 50% (Cardno and Gottesman, 2000). This lack of complete concordance in monozygotic twins suggests that schizophrenia is caused not

only due to dysfunctional genes but also due to gene activation or silencing by the environmental factors (Stahl, 2013). The environmental factors that increases the risk for schizophrenia include maternal malnutrition during famine, maternal infections (e.g., rubella, Toxoplasma and herpes simplex virus type 2), maternal obstetric complications (e.g., placental pathology, diabetes, rhesus incompatibility, pre-eclampsia or bleeding), abnormal foetal development (e.g., low birth weight, congenital malformations or reduced head circumference), delivery complications (e.g., uterine atony, asphyxia or emergency caesarean section), season of birth, advanced paternal age, childhood trauma, social factors (e.g., parental separation or loss, social exclusion), urban environment, migration, and substance abuse (Stilo et al., 2011). These environmental factors might activate or silence a gene through epigenetic modifications such as DNA methylation, histone modifications, and microRNAs leading to the development of schizophrenia (Feil and Fraga, 2012; Alelú-Paz et al., 2016).

3.3.3 Gene-environment interplay

Schizophrenia is caused by both nature (i.e., genetics) and nurture (i.e., environment or epigenetics) (Rapoport et al., 2012; Stahl, 2013). During brain development, multiple genetic risk factors for schizophrenia might interact with a wide range of environmental insults triggering a complex cascade of pathophysiological process such as gene and protein expression impairment leading to changes in neuro-circuits resulting in inefficient information processing which ultimately manifests as behavioural, cognitive, and emotional deficits of the disorder (Horváth and Mirnics, 2009; Pishva et al., 2014).

3.4 Stages of schizophrenia

The psychotic features of schizophrenia emerge in late adolescence or early adulthood, with a peak between ages 18 and 25 years (Insel, 2010). The onset of psychotic features of schizophrenia is preceded by a broad range of symptoms. A clinical staging of schizophrenia helps in mapping out the whole spectrum of the disorder from earliest signs to chronic psychosis, thus providing a guide for prevention and specialized treatment (Fava and Kellner, 1993; McGorry, 1995; McGorry et al., 2006). In the handful of clinical staging models proposed for schizophrenia, the pre-psychotic phase of the disorder was taken as a single clinical entity known as prodrome which was a heterogeneous group (Fava and Kellner, 1993; Lieberman et al., 2001). Subsequently, McGorry and colleagues proposed a model that underwent various updates which attempted to subdivide the pre-psychotic phase of the disorder into homogenous subgroups (McGorry, 1995; McGorry et al., 2006; Agius et al., 2010; McGorry

et al., 2010, 2014; McGorry and Nelson, 2016). The McGorry model comprises of five stages, namely stage 0: increased risk of psychotic disorder with no current symptoms, stage 1a: mild/nonspecific symptoms, stage 1b: moderate subthreshold symptoms, stage 2: onset of the first episode of psychosis, stage 3a: incomplete remission from the first episode of care, stage 3b: recurrence or relapse of the psychotic disorder, stage 3c: multiple relapses when worsening in clinical extent and objective presence of the impact of the disorder, and stage 4: severe, persistent or unremitting disorder (McGlashan et al., 2010). The clinical high risk for psychosis (CHR) state includes individuals in McGorry's stages 1 and 2. A recent study proposed a 4-stage model for psychosis prodrome, which is based on the notion that increased symptom severity leads to an increased risk of developing psychosis: stage 1: primarily negative type symptoms and early risk factors with the absence of attenuated positive symptoms, stage 2: mild to moderate attenuated form of positive symptoms, stage 3: severe attenuated form of positive symptoms, and stage 4: presence of only one positive symptom of psychotic intensity (Carrión et al., 2016). These clinical staging of schizophrenia has improved the possibility of early identification and intervention at the stage of CHR for psychosis to limit the progression of the disorder.

Individuals at CHR for psychosis are identified by two complementary approaches, namely the high risk (HR) and the ultra-high risk (UHR) criteria. The HR criteria based on basic symptoms, which comprises of two partially overlapping risk entities, the cognitive-perceptive basic symptoms (COPER) and the cognitive disturbances (COGDIS) (Schultze-Lutter et al., 2012, 2015). The UHR criteria include attenuated positive symptoms syndrome (APSS), brief limited intermittent psychotic symptoms (BLIPS), and a combination of a risk factor for psychosis and a recent functional decline (McGlashan et al., 2010). Both these approaches have been used in the early recognition of psychosis studies to predict the transition rate to schizophrenia (Gee and Cannon, 2011; Fusar-Poli et al., 2012).

3.5 Neurodevelopmental model of schizophrenia

Schizophrenia is hypothesized as an end stage of abnormal neurodevelopmental processes that began years before the actual onset of the disorder, which is why it is widely accepted as a neurodevelopmental disorder (Insel, 2010; Rapoport et al., 2012). Despite many years of research in the field of schizophrenia, the complex factors preceding the onset of psychosis is not yet completely understood. Normal cortical development involves proliferation, migration, arborization (circuit formation), and myelination. Proliferation and migration occur mostly during the prenatal period, whereas arborization and myelination also occur

throughout the first two decades of postnatal life (Insel, 2010). Longitudinal neuroimaging studies in healthy controls have demonstrated that changes in grey-matter density occur until mid-twenties with the prefrontal cortex being the last to mature (Figure 1a) (Paus et al., 2008). This progressive reduction of grey-matter density is thought to be due to combined effects of pruning of neuronal circuits and deposition of myelin (Insel, 2010). During adolescence and early adulthood, there is an increase in inhibitory and decrease in excitatory synapses in the prefrontal cortex (Figure 1a). It has been hypothesised that this trajectory of cortical development is defective i.e., reduced elaboration of inhibitory pathways and excessive pruning of excitatory pathways leading to altered excitatory-inhibitory balance in the prefrontal cortex of individuals who develop schizophrenia (Figure 1b) (Insel, 2010).

The Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) Consortium has analysed neuroimaging data from approximately 2,000 schizophrenia patients collected from 17 research groups around the world as part of the ENIGMA Schizophrenia Working Group (Thompson et al., 2014; van Erp et al., 2016; Walton et al., 2017a,b). These meta-analyses found that schizophrenia patients had significantly smaller hippocampus, amygdala, thalamus, nucleus accumbens, and intracranial volumes as well as larger pallidum and lateral ventricle volumes compared to healthy controls (van Erp et al., 2016), positive symptoms were related to cortical thinning in the superior temporal gyrus (Walton et al., 2017a), and negative symptoms were related to cortical thinning in the medial orbitofrontal cortex (Walton et al., 2017b). The ENIGMA Schizophrenia Working Group is actively working on analysing longitudinal neuroimaging data in first-episode schizophrenia patients that might help in understanding defective cortical development trajectories hypothesised in the neurodevelopmental model of schizophrenia.

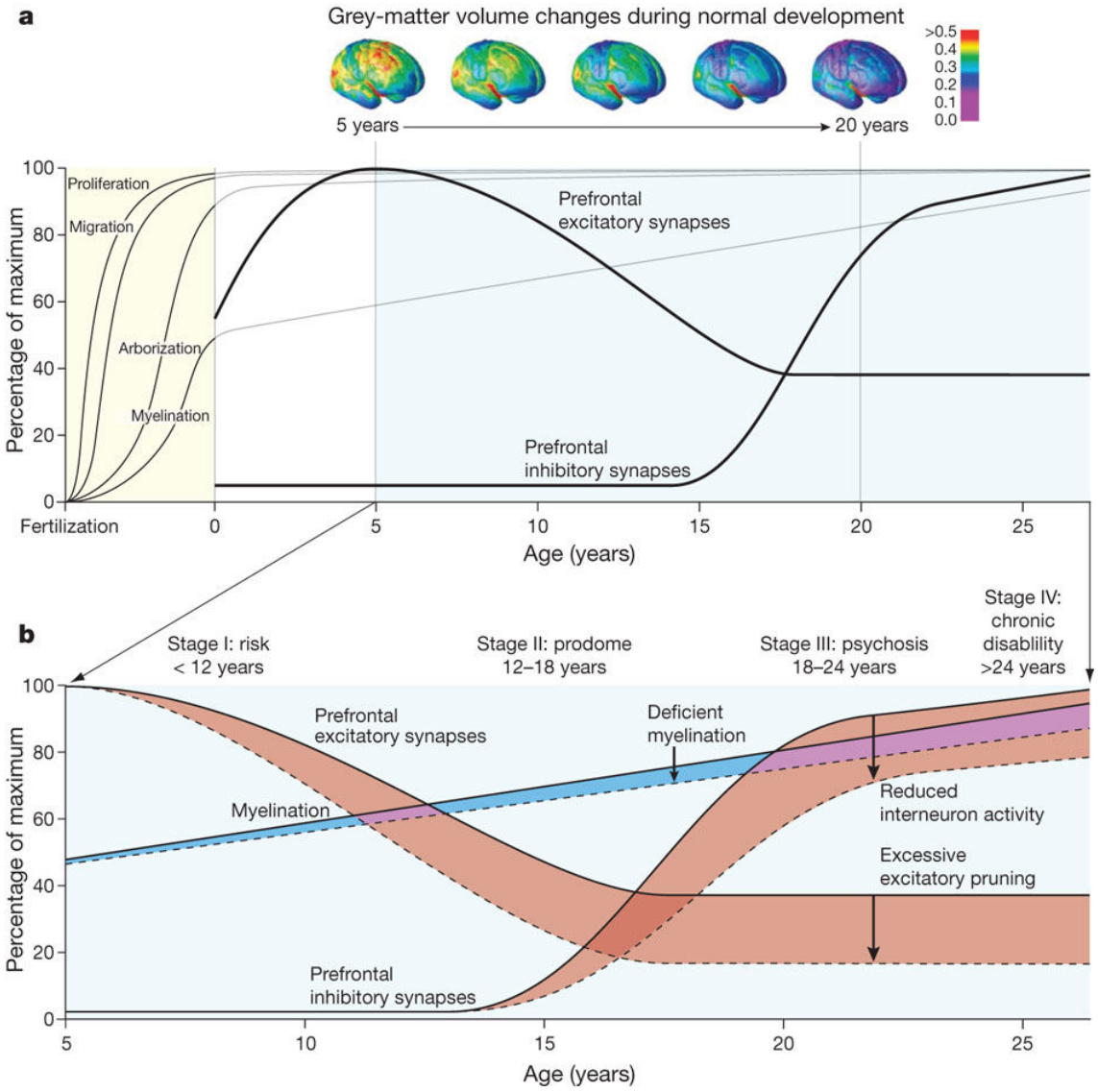

Figure 1: Neurodevelopmental model of schizophrenia. (**a**) Normal cortical development in humans from prenatal period until 25 years of age, and (**b**) Defective cortical development from ages 5 to 25 years in children who transition to schizophrenia (Insel, 2010).

3.6 Neurochemical hypotheses of schizophrenia

Schizophrenia is a multifactorial psychiatric disorder, but the exact cause of the disorder remains unclear. Dysfunctions in neurotransmitter pathways such as dopamine, glutamate, gamma-aminobutyric acid (GABA), and serotonin have been implicated in the pathophysiology of schizophrenia (Eggers, 2013; Brisch et al., 2014; Hu et al., 2015; Chang et al., 2016), which will be explained further in the following sections.

3.6.1 Dopamine hypothesis of schizophrenia

The dopamine hypothesis was proposed based on two fundamental observations. First, the psychostimulants such as amphetamine and cocaine

that blocked the reuptake of dopamine, and increased dopamine at the synaptic cleft induced positive symptoms of schizophrenia in healthy volunteers and exacerbated these symptoms in schizophrenia patients (Bunney et al., 1973; Lieberman et al., 1987; Einhorn et al., 1988; Prus, 2017; Satel and Edell, 1991). Secondly, the antipsychotic medications that block dopamine D2 receptors relieved the positive symptoms of schizophrenia (Creese et al., 1976; Peroutka, 1980; Kapur et al., 2000). There are four main dopamine pathways in the brain, namely mesolimbic (from ventral tegmental area in the brainstem to nucleus accumbens in the ventral striatum), mesocortical (from ventral tegmental area to prefrontal cortex), nigrostriatal (from substantia nigra to basal ganglia or striatum), and tuberoinfundibular (from hypothalamus to anterior pituitary) (Stahl, 2013). The two key dopamine pathways implicated in the pathology of schizophrenia are mesolimbic and mesocortical pathways. The dopamine hypothesis states that the hyperactivity of dopamine neurons in the mesolimbic pathway mediate positive symptoms of schizophrenia, and hypoactivity of dopamine neurons in the mesocortical pathway mediate negative symptoms and cognitive impairment in schizophrenia (Stahl, 2013).

3.6.2 Serotonin hypothesis of schizophrenia

Involvement of serotonergic pathways in the pathophysiology of schizophrenia was based on the observation that lysergic acid diethylamide (LSD), a 5-hydroxytryptamine (5-HT) serotonin receptor agonist, induced or exacerbated schizophrenia-like symptoms in humans (Roth and Meltzer, 1995). The hypothesis states that schizophrenia is caused by the chronic widespread stress-induced serotonergic overdrive in the cerebral cortex, especially in the anterior cingulate cortex (ACC) and dorsolateral frontal lobe. This serotonergic overdrive might lead to disruption in glutamate signalling ultimately leading to synaptic atrophy and grey matter loss. Positive symptoms might be caused by normal dopamine input to impaired ACC, and negative symptoms and cognitive impairment might be caused by frontal lobe hibernation (Eggers, 2013).

3.6.3 Glutamate hypothesis of schizophrenia

The glutamate hypothesis of schizophrenia was proposed based on the observation that N-methyl-D-Aspartate (NMDA) receptor antagonists such as phencyclidine and ketamine produced positive symptoms, negative symptoms, and cognitive deficits very similar to symptoms of schizophrenia in healthy volunteers, and also exacerbated all these symptoms in schizophrenia patients (Javitt, 1987; Javitt et al., 1991; Krystal et al., 1994; Lahti et al., 1995; Malhotra et al., 1997; Umbricht et al., 2000). Antipsychotics, dopamine D2 receptor

antagonists are effective against positive symptoms but have little effect on negative symptoms and cognitive deficits (Javitt, 2010). In a study comparing the effect of amphetamine (dopamine reuptake blocker) and ketamine (NMDA receptor antagonist) in healthy controls, the authors reported that both these drugs induce positive symptoms, however, only ketamine induces negative symptoms and cognitive deficits (Krystal et al., 2005). Furthermore, individuals with NMDA receptor autoimmune encephalitis (antibodies against NR1A subunit) present with symptoms similar to schizophrenia (Dalmau et al., 2007, 2008, 2011). Lastly, meta-analyses have reported that NMDA receptor agonists D-serine and glycine, and glycine transporter type 1 inhibitor sarcosine reduce total and negative symptoms as an adjunct to antipsychotic medications (Singh and Singh, 2011). Based on all these findings, the glutamate hypothesis is thought to explain the negative symptoms and cognitive deficits better than the dopamine hypothesis.

The glutamate hypothesis is also known as NMDA receptor hypofunction hypothesis of schizophrenia. There are several glutamate pathways in the brain. The cortico-brainstem glutamate pathway projects from cortical pyramidal neurons in the prefrontal cortex to brainstem neurotransmitter centres (raphe nucleus, locus coeruleus, ventral tegmental area, substantia nigra). This glutamate pathway is hypothesised to be a key pathway in the pathology of schizophrenia as it can regulate dopamine neurons in the ventral tegmental area (Stahl, 2013). The hypoactive NMDA receptors on cortical GABA interneurons of the cortico-brainstem glutamate pathway might cause excessive glutamate release in the ventral tegmental area leading to hyperactivity of mesolimbic dopamine pathway mediating positive symptoms of schizophrenia. The hypoactive NMDA receptors on cortical GABA interneurons of the cortico-brainstem glutamate pathway might cause excessive glutamate release in the ventral tegmental area leading to activation of GABA interneurons which in turn inhibits the mesocortical dopamine neurons mediating negative symptoms and cognitive dysfunction in schizophrenia. Thus, the glutamate neurons innervate mesolimbic dopamine neurons directly leading to mesolimbic hyperactivity, whereas they innervate mesocortical dopamine neurons indirectly via GABA interneurons leading to mesocortical hypoactivity (Stahl, 2013). As the current thesis mainly focusses on the glutamate hypothesis based on the NMDA receptor hypofunction, the molecular basis of this theory will be discussed in the next section.

3.7 Molecular basis of NMDA receptor hypofunction in schizophrenia

3.7.1 N-methyl-D-aspartate (NMDA) receptors

NMDA receptors are voltage dependent ionotropic glutamate receptors. To date, seven different NMDA receptor subunits belonging to three subfamilies are identified in humans, namely one NR1 subunit, four different NR2 subunits (NR2A, NR2B, NR2C, NR2D), and two NR3 subunits (NR3A, NR3B). Functional NMDA receptors are heterotetramers composed of two obligatory glycine-binding NR1 subunits combined with two regulatory glutamate-binding NR2 subunits (Collingridge et al., 2009; Paoletti et al., 2013). NMDA receptors are organised into amino-terminal domains and ligand-binding domains on the extracellular side of the membrane, a transmembrane domain spanning the membrane which defines the ion channel pore, and an intracellular carboxy-terminal domain within the cytoplasm (Figure 2) (Traynelis et al., 2010; Lee et al., 2014). NMDA receptors are blocked by magnesium at resting state, which prevents calcium influx. NMDA receptors are Hebbian-like coincidence detectors, which require three events to occur at the same time: glutamate binding to the NR2 subunit, glycine/D-serine binding to the NR1 subunit, and membrane depolarization to remove magnesium block that open an ion conductive pore to let calcium into a neuron to trigger post-synaptic glutamatergic neurotransmission (Mayer et al., 1984; Nowak et al., 1984; Lee et al., 2014). This NMDA receptor mediated calcium signalling plays a crucial role in neural development and synaptic plasticity (Lau et al., 2009).

3.7.2 D-serine

D-serine, a co-agonist of NMDA receptors, is a D-amino acid. It is found at much higher concentrations in the brain than in the periphery. It is synthesised from L-serine by the pyridoxal phosphate (PLP) dependent serine racemase (SRR) enzyme and is presumed to be degraded by both SRR and D-amino acid oxidase (DAO/DAAO) enzymes (Figure 3a) (Wolosker et al., 2008; Sacchi, 2013). D-serine is a co-agonist of NMDA receptors, which binds to the strychnine-insensitive glycine modulatory site on the NR1 subunit of the NMDA receptors (Figure 2) (Mothet et al., 2000). D-serine was found to have three times higher affinity to NMDA receptors than glycine. This higher affinity of D-serine than glycine was explained through a crystallography experiment which found D-serine displaces a water molecule from the NR1 binding site and makes three additional hydrogen bonds with this site of the NMDA receptors (Furukawa and Gouaux, 2003).

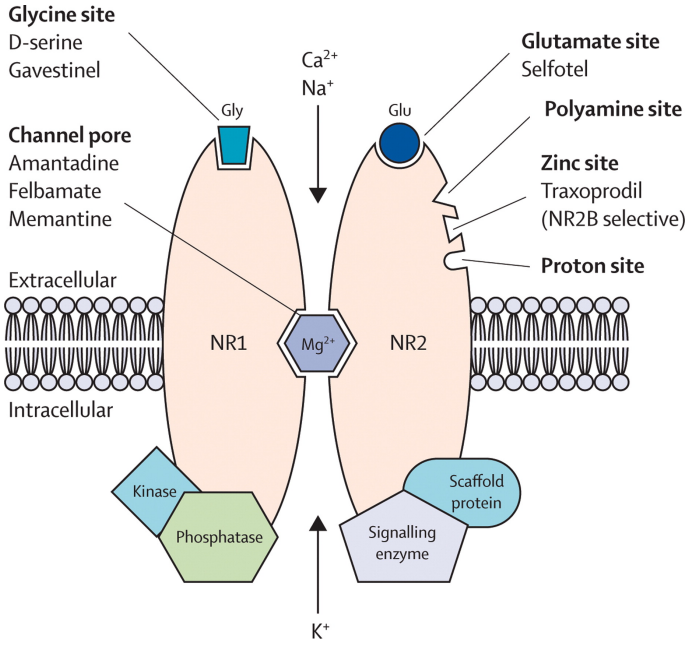

Figure 2: NMDA receptor complex. An extracellular domain includes binding sites for glycine or D-serine on the NR1 subunit, and glutamate on the NR2 subunit, a transmembrane domain defining the ion-channel pore blocked by magnesium, and an intracellular domain for binding of signalling molecules (Kalia et al., 2008).

3.7.3 D-amino acid oxidase (DAO)

The human *DAO* gene is located at the chromosome 12q24, which comprises of eleven exons. It encodes for a ∼39 kDa protein of 347 amino acids (Verrall et al., 2010). This gene has been shown to have 8 splice variants, out of which only 4 variants have been shown to be protein coding, that encodes for proteins of sizes ∼39 kDa, ∼31 kDa, ∼19 kDa, and ∼22 kDa (Uhlén et al., 2015; McLaren et al., 2016).

DAO is a flavoenzyme, which catalyses the oxidation of D-amino acids through concomitant reduction of flavin adenine dinucleotide (FAD), producing the corresponding imino acid, which is further hydrolyzed to yield ammonia and corresponding α-keto acid. During FAD re-oxidation, hydrogen peroxide is produced (Verrall et al., 2010). FAD-bound DAO was found to be active, whereas FAD-unbound DAO was found to be inactive (Caldinelli et al., 2009; Terry-Lorenzo et al., 2014). FAD binding is weaker in human DAO compared to DAO from other species, which provides human DAO with a potential means to regulate DAO activity in humans (Caldinelli et al., 2009). DAO oxidises D-amino acids with small, neutral side chains such as D-serine, D-alanine, D-proline, and D-Leucine (Molla et al., 2006b; Verrall et al., 2010). The main substrate of DAO in the brain is D-serine as it is by far the most abundant D-amino acid in the brain (Bendikov et al., 2007; Verrall et al., 2010).

DAO is widely distributed in the human central nervous system (CNS). DAO is generally considered as a hindbrain enzyme, which is enriched in the cerebellum and the brainstem (Sacchi et al., 2012). DAO activity was robustly detected in the human cerebellum (Kapoor et al., 2006). However, DAO activity has been detected in the human forebrain in some studies (Madeira et al., 2008; Sasabe et al., 2014), albeit only at a small fraction compared to the human cerebellum (Verrall et al., 2010). DAO mRNA and protein are mainly detected in the human liver, kidney, and brain (Verrall et al., 2007; GTEx Consortium, 2015; Uhlén et al., 2015). In the human brain, both DAO mRNA and protein are highly expressed in the cerebellum than in other brain regions (GTEx Consortium, 2015; Uhlén et al., 2015). Furthermore, DAO is not solely present in glial cells, but there is evidence that DAO is also present in neurons (Verrall et al., 2007, 2010). Initially, DAO was thought to be present only in peroxisomes (De Duve and Baudhuin, 1966), however presence of extra-peroxisomally active DAO has been reported both in glial cells (Sacchi et al., 2008, 2011) and neurons (Popiolek et al., 2011).

3.7.4 D-amino acid oxidase activator (DAOA/G72)

The human DAO activator (*DAOA/G72*) is a primate specific gene, which is located at chromosome 13q33 and comprises of seven exons (Sacchi et al., 2016). It encodes for a ∼20 kDa protein of 153 amino acids (Benzel et al., 2008). This gene has been shown to have 12 splice variants, out of which only 4 variants have been shown to be protein coding, that encodes for proteins of sizes ∼20 kDa, ∼20 kDa, ∼10 kDa, and ∼15 kDa (Uhlén et al., 2015; McLaren et al., 2016).

Several lines of evidence suggest that DAOA interacts with DAO. However, the effect of DAOA on DAO has been controversial to date. Some studies have found DAOA to be an activator of DAO (Chumakov et al., 2002; Chang et al., 2013). However, in another study, DAOA was found to be an inhibitor of DAO (Sacchi et al., 2008). A study also found no effect of DAOA on DAO (Kvajo et al., 2008). Therefore, future studies with physiological levels of DAOA and DAO will be required to conclusively comment whether DAOA is an activator or inhibitor of DAO.

The expression pattern of DAOA mRNA and protein in the human brain is not yet fully characterised. Only earlier studies have detected *DAOA* mRNA in the human brain (Chumakov et al., 2002; Korostishevsky et al., 2006). However, recent studies could not replicate these findings of *DAOA* mRNA expression in the human brain (Benzel et al., 2008; GTEx Consortium, 2015). Furthermore, DAOA protein was detected in the human cerebellum and the cerebral cortex (Uhlén et al., 2015), and in the human amygdala (Kvajo et al., 2008). On the

contrary, a study could not detect DAOA protein in these human brain regions (Benzel et al., 2008). DAOA protein has been shown to localise in mitochondria and cause mitochondrial dysfunction (Kvajo et al., 2008; Sacchi et al., 2011; Otte et al., 2014).

3.7.5 Genetic association of *DAO* and *DAOA* with schizophrenia

A landmark study (Chumakov et al., 2002) identified *DAO* and *DAOA* as putative risk genes for schizophrenia. This study identified four *DAO* SNPs (rs211902, rs3918346, rs3741775, rs3918347, all intronic) and six *DAOA* SNPs (rs3916965, rs3916967, rs2391191, rs778293, rs3918342, rs1421292) to be associated with schizophrenia. Subsequently, these and many other *DAO* and *DAOA* SNPs have been examined in many case-control and family association studies of schizophrenia. These studies were included in the SchizophreniaGene (SzGene) meta-analysis, which showed a significant association of 24 genetic variants in 16 different genes with schizophrenia (Allen et al., 2008). In this meta-analysis, one *DAO* SNP (rs4623951) and two *DAOA* SNPs (rs778293, rs3916971) were significantly associated with schizophrenia (Allen et al., 2008). A mega-analysis conducted by the PGC combining GWAS data from 17 separate studies with a total of 9,394 cases and 12,462 controls identified seven significant SNP loci for schizophrenia, but none of the loci were within *DAO* and *DAOA* gene regions (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). A recent GWAS by the PGC involving 37,000 cases and 113,000 controls identified around 108 schizophrenia associated loci, and again none were within *DAO* and *DAOA* gene regions with genome-wide significance ($p \leq 5\times10^{8}$). Nevertheless, in this GWAS, *DAO* SNPs (rs2111902, rs3918346, rs3741775, rs3918347, rs2070586, and rs2070587) showed nominal association ($p<0.05$) with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Therefore, *DAO* and *DAOA* remain as candidate genes for schizophrenia because of their role in glutamatergic signalling.

3.7.6 Roles of DAO and DAOA in NMDA receptor hypofunction

The NMDA receptor hypofunction theory proposed in schizophrenia is based on the observations in schizophrenia patients of increased DAO activity leading to decreased D-serine which in turn results in NMDA receptor hypofunction (Figure 3b). Human post-mortem studies have shown that there is increased DAO activity in the cerebellum of schizophrenia patients compared to healthy controls (Kapoor et al., 2006; Burnet et al., 2008). There is also evidence for decreased D-serine in serum and cerebrospinal fluid (CSF) of schizophrenia

patients compared to healthy controls (Hashimoto et al., 2003, 2005; Bendikov et al., 2007; El-Tallawy et al., 2017). There is post-mortem evidence for a significant decrease in NMDA receptor subunits, mainly NR1 mRNA and protein in the prefrontal cortex of schizophrenia patients compared to controls (Weickert et al., 2013; Catts et al., 2016). A study found increased DAOA mRNA and protein levels in schizophrenia brains (Korostishevsky et al., 2006), which wasn't replicated in the subsequent study (Benzel et al., 2008). Furthermore, studies have reported increased DAOA protein levels in the plasma of schizophrenia patients compared to healthy controls (Lin et al., 2014; Akyol et al., 2017), which was not replicated in a recent study (Ishiwata et al., 2017). Thus, the effect of DAOA on DAO activity and in general the role of DAOA in schizophrenia is yet to be elucidated.

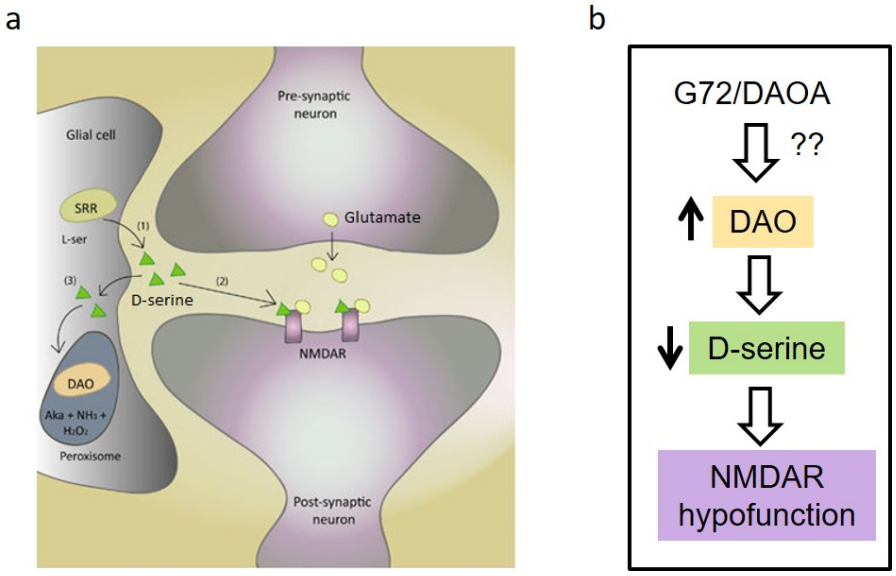

Figure 3: Molecular basis of NMDA receptor hypofunction theory proposed in schizophrenia. (**a**) A normal tripartite synapse showing synthesis of D-serine (green triangles) from L-serine (L-ser) by serine racemase (SRR), degradation of D-serine by DAO into alpha-keto acid (Aka), ammonia (NH_3), hydrogen peroxide (H_2O_2), and NMDA receptors (NMDAR) requiring both D-serine and glutamate (yellow circles) for glutamatergic neurotransmission (Verrall et al., 2010). (**b**) Proposed NMDAR hypofunction molecular cascade in schizophrenia showing increased DAO activity leading to decreased D-serine, and finally NMDAR hypofunction, and an unclear effect of DAOA/G72 on DAO.

3.8 DAO and DAOA as targets for the treatment of schizophrenia

The current antipsychotics that are dopamine D2 receptor antagonists used in the treatment of schizophrenia are more effective against positive symptoms than against negative symptoms and cognitive impairment in schizophrenia (Lally and MacCabe, 2015). Thus, there is an urgent need to develop novel

and more efficacious medications to ameliorate negative symptoms and cognitive impairment in schizophrenia. In the search for developing novel drugs for schizophrenia, many studies have focussed on testing NMDA receptor modulators such as D-serine and glycine as adjunctive therapy to the ongoing antipsychotic treatment (Woods et al., 2013). The use of D-serine and glycine is limited by the high doses needed for efficacy, and also nephrotoxicity caused by D-serine (Williams and Lock, 2004). To circumvent the nephrotoxicity of D-serine, DAO inhibitors that inhibit the DAO activity leading to increase in D-serine have been tested as a novel treatment for schizophrenia. The DAO inhibitor 5-chloro-benzo[d]isoxazol-3-ol (CBIO) administered with D-serine has been reported to enhance D-serine efficacy in mice (Hashimoto et al., 2009), but a recent study couldn't replicate this finding (Sershen et al., 2016). Recently, sodium benzoate has been tested both in preclinical (Matsuura et al., 2015) and clinical studies (Lane et al., 2013; Lin et al., 2017) with encouraging results. However, the affinity of sodium benzoate to DAO is low and the extent to which the effects were mediated by DAO inhibition remains unclear (Sershen et al., 2016). To date, no modulators of DAOA has been developed as a novel treatment for schizophrenia. These unsuccessful attempts in the development of novel treatment for schizophrenia highlight that the interplay between DAOA, DAO, D-serine, and NMDA receptors is not yet completely understood. Therefore, there is a need to elucidate this pathway with the hope that DAO or DAOA might emerge as therapeutic targets in future.

4. Aims of the thesis

DAO and *DAOA* are putative schizophrenia susceptibility genes. These genes are hypothesised to interact with each other to cause NMDA receptor hypofunction in schizophrenia. Despite many years of research in the field of schizophrenia, the exact function of DAOA, its expression in the normal human brain, and its interaction with DAO still needs to be elucidated (Sacchi et al., 2016). The overall objectives of this thesis were to understand DAO and DAOA trajectory expression in the normal human brain, to re-evaluate the association of *DAO* and *DAOA* SNPs with schizophrenia using meta-analysis, to assess whether *DAO* and *DAOA* SNPs predict transition to schizophrenia and endophenotypes in individuals at clinical risk for psychosis, and to determine the interaction between DAO and DAOA in human cell lines.

Study I: Expression of D-Amino Acid Oxidase (*DAO*/*DAAO*) and D-Amino Acid Oxidase Activator (*DAOA*/*G72*) during development and aging in the human post-mortem brain

In this study, we used human post-mortem brain samples from 55 subjects across six brain regions (cerebellum, brainstem, amygdala, thalamus, striatum, and frontal cortex) with ages ranging from 16 weeks of gestation to 91 years, who had no history of neuropsychiatric disorder and their brain tissues showed no neuropathological evidence of any neuropsychiatric disorder. The main aims of this study were as follows:

(a) To determine DAO and DAOA mRNA and protein expression in the normal human brain during development and aging to understand the concomitant expression of DAO and DAOA in different brain regions.

(b) To assess the effect of *DAO* and *DAOA* SNPs on DAO and DAOA expression levels to elucidate the role of these SNPs in the regulation of DAO and DAOA expression.

(c) To determine DNA methylation levels at *DAO* and *DAOA* CpG sites in the cerebellum and the frontal cortex of control human post-mortem brain *in silico* to understand the regulation of DAO and DAOA expression at the transcription level.

To address these aims, we quantified *DAO* and *DAOA* mRNA levels using quantitative real-time reverse transcription-PCR (qRT-PCR), and DAO and DAOA protein levels using commercially available DAO and DAOA

enzyme-linked immunosorbent assay (ELISA) kits. We assessed *DAO* (rs3918347, rs4623951) and *DAOA* (rs778293, rs3916971, rs746187) SNPs using TaqMan SNP genotyping assay. *In silico* DNA methylation levels at *DAO* and *DAOA* CpG sites were downloaded from Gene Expression Omnibus (GEO) datasets.

Study II: A systematic meta-analysis of the association of Neuregulin 1 (*NRG1*), D-amino acid oxidase (*DAO*), and DAO activator (*DAOA*)/*G72* polymorphisms with schizophrenia

The aim of this study was to perform an up to date systematic meta-analysis of both previously meta-analysed as well as new schizophrenia association studies to comprehensively evaluate the association of 8 *DAO* and 12 *DAOA* SNPs with schizophrenia. To address this, we conducted systematic literature search which resulted ultimately in 20 *DAO* and 23 *DAOA* case-control schizophrenia association studies that fulfilled the inclusion criteria. Meta-analyses of these studies were conducted using the MIX 2.0-Professional software for meta-analysis in Excel, version 2.0.1.4.

Study III: Prediction analysis for transition to schizophrenia in individuals at clinical high-risk for psychosis: The relationship of *DAO*, *DAOA* and *NRG1* variants with negative symptoms and cognitive deficits

In this study, we used whole blood samples from 185 individuals at clinical risk for psychosis who were followed-up at 6, 12, 24, and 36 months for the transition to schizophrenia-spectrum disorders. The main aims of this study were as follows:

(a) To determine *DAO* and *DAOA* SNPs and mRNA levels in at-risk individuals to identify predictive genetic markers in these individuals for the transition to schizophrenia-spectrum disorders.

(b) To identify endophenotypes in at-risk individuals using psychopathology constructs according to Research Domain Criteria (RDoC) guidelines.

To address these aims, we quantified *DAO* and *DAOA* mRNA levels using qRT-PCR and assessed *DAO* (rs3918347, rs4623951) and *DAOA* (rs778293, rs3916971, rs746187) SNPs using TaqMan SNP genotyping assay.

Study IV: Controversial effects of D-amino acid oxidase activator (DAOA)/G72 on D-amino acid oxidase (DAO) activity in human neuronal, astrocyte, and kidney cell lines: The N-methyl D-aspartate (NMDA) receptor hypofunction point of view

In this study, we used human neuroblastoma SH-SY5Y cells, human astrocytoma cells 1321N1 cells, and human embryonic kidney HEK293 cells. The aims of this study were as follows:

(a) To measure DAO activity in DAO and DAOA co-transfected SH-SY5Y, 1321N1, and HEK293 cells to understand the effect of DAOA on DAO activity.

(b) To measure NMDA receptor activity in HEK293 cells stably transfected with NMDA receptor subunits NR1 and NR2A to determine the effect of DAOA on NMDA receptor activity.

(c) To simulate the DAO and DAOA interactions to evaluate the effect of DAOA on DAO *in silico*.

To address these aims, the human cell lines were co-transfected with DAO and DAOA plasmid constructs, DAO activity was measured based on the release of hydrogen peroxide and its interaction with an Amplex Red reagent, NMDA receptor activity was measured using patch-clamp whole-cell recording, and *in silico* experiments were performed using Molecular Dynamics simulations.

5. Results

5.1 Study I: Expression of D-Amino Acid Oxidase (*DAO*/*DAAO*) and D-Amino Acid Oxidase Activator (*DAOA*/*G72*) during development and aging in the human post-mortem brain

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Author contributions

ZM, C-MM, and EG designed the experiments. VJ performed experiments, analysed data, and wrote the manuscript. ZM, C-MM, SW, and EG reviewed and revised the manuscript. All authors have approved the final manuscript.

5.1.1 Abstract

In the brain, D-amino acid oxidase (DAO/DAAO) mainly oxidizes D-serine, a co-agonist of the N-methyl-D-aspartate (NMDA) receptors. Thus, DAO can regulate the function of NMDA receptors via D-serine breakdown. Furthermore, DAO activator (DAOA)/G72 has been reported as both DAOA and repressor. The co-expression of DAO and DAOA genes and proteins in the human brain is not yet elucidated. The aim of this study was to understand the regional and age span distribution of DAO and DAOA (mRNA and protein) in a concomitant manner. We determined DAO and DAOA mRNA and protein expression across six brain regions in normal human post-mortem brain samples (16 weeks of gestation to 91 years) using quantitative real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay. We found higher expression of *DAO* mRNA in the cerebellum, whereas lower expression of DAO protein in the cerebellum compared to the other brain regions studied, which suggests post-transcriptional regulation. We detected DAOA protein but not *DAOA* mRNA in all brain regions studied, suggesting a tightly regulated expression. To understand this regulation at the transcriptional level, we analyzed DNA methylation levels at *DAO* and *DAOA* CpG sites in the cerebellum and frontal cortex of control human post-mortem brain obtained from Gene Expression Omnibus datasets. Indeed, *DAO* and *DAOA* CpG sites in the cerebellum were significantly more methylated than those in the frontal cortex. While investigating lifespan effects, we found that *DAO* mRNA levels were positively correlated with age <2 years in the cerebellum and amygdala. We also detected a significant positive correlation (controlled for age) between DAO and DAOA protein in all of the brain regions studied except for the frontal cortex. In summary, DAO and DAOA expression in the human brain are both age and brain region dependent.

5.1.2 Introduction

Brain development is a continuous process, which extends from the prenatal period into adulthood (Rapoport et al., 2012). Abnormal neurodevelopmental processes are implicated in many psychiatric disorders such as schizophrenia, attention deficit hyperactivity disorder, autism spectrum disorder, and intellectual disability (Rapoport et al., 2012; Miller et al., 2016). Moreover, altered gene expression across different brain regions and lifespan has been reported in psychiatric disorders (Sibille, 2013; Darby et al., 2016). For example, schizophrenia risk genes are reported to be associated with transcripts that are either enriched in specific brain regions or unique to the human brain, and some also show preferential expression in the fetal brain (Kang et al., 2011; Kleinman et al., 2011). Therefore, it is important to analyze the brain region-specific expression of risk genes for psychiatric disorders in the normal human brain across the lifespan to understand the mechanisms underlying pathological changes in psychiatric disorders.

D-amino acid oxidase (DAO/DAAO; from now on addressed as DAO) is a flavoenzyme, which oxidizes D-amino acids. Its main substrate in the brain is D-serine (Pollegioni et al., 2007; Sacchi et al., 2012). D-serine is a co-agonist of N-methyl-D-aspartate (NMDA) receptors. In addition to glutamate, NMDA receptors require a co-agonist (glycine or D-serine) binding at the glycine modulatory site in order to function normally. D-serine is shown to be more potent in binding to NMDA receptors than glycine (Matsui et al., 1995; Shleper et al., 2005; Sacchi et al., 2012). Thus, DAO can regulate the function of NMDA receptors via D-serine breakdown, which may lead to NMDA receptor hypofunction (Kantrowitz and Javitt, 2010; Labrie and Roder, 2010; Verrall et al., 2010; Labrie et al., 2012). One of the possible explanations for NMDA receptor hypofunction theory proposed in schizophrenia is probably an increased activity of DAO leading to decreased D-serine. D-amino acid oxidase activator (DAOA)/G72 (from now on addressed as DAOA) binds to DAO, but the effect of DAOA on DAO is controversial. This is because DAOA is reported to both increase (Chumakov et al., 2002; Chang et al., 2013) and decrease (Sacchi et al., 2008, 2011) the activity of DAO. DAOA is localized in mitochondria and reported to modulate its function (Kvajo et al., 2008; Sacchi et al., 2011; Otte et al., 2014). Thus, the exact function of DAOA is not yet completely understood.

The human *DAO* gene located on chromosome 12q24 encodes for a ∼39 kDa protein (Almond et al., 2006; Sacchi et al., 2008; Verrall et al., 2010) and the human *DAOA* gene encodes for a ∼20 kDa protein (Benzel et al., 2008). The genes *DAOA/G30* overlap and are transcribed from opposite strands on

chromosome 13q33. The polymorphisms of these genes in non-coding regions have been described to be associated with the pathophysiology of schizophrenia and bipolar disorder (Detera-Wadleigh and McMahon, 2006; Korostishevsky et al., 2006; Corvin et al., 2007b; Prata et al., 2008; Mössner et al., 2010; Labrie et al., 2012; Gatt et al., 2015; Liu et al., 2016). However, a recent genome-wide association study (GWAS) conducted by the Psychiatric Genomics Consortium (PGC) found that out of 108 schizophrenia-associated loci, none were within *DAO* and *DAOA* gene regions (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). There are several lines of evidence showing regional and cellular expression of DAO mRNA and protein in the human brain (Kapoor et al., 2006; Verrall et al., 2007; Habl et al., 2009; Ono et al., 2009), however, none of these studies isolated mRNA and protein concomitantly from the same brain tissue as we did in this study. On the other hand, the reports of DAOA mRNA and protein expression in the human brain remain unconvincing to date. Studies showing *DAOA* mRNA expression in the human brain are limited (Chumakov et al., 2002; Korostishevsky et al., 2006). Moreover, the expression of DAOA mRNA and protein in the human brain has been questioned (Benzel et al., 2008; GTEx Consortium, 2015). The human protein atlas detected DAOA protein (also known as pLG72, from now on addressed as DAOA) in the cerebellum and the cerebral cortex (Uhlén et al., 2015). Thus, the regulation of DAO and DAOA expression in the human brain has not yet been elucidated.

Furthermore, since DNA methylation can lead to both increases and decreases in gene expression (Wagner et al., 2014), it may play a crucial role in the regulation of normal brain development (Fan et al., 2001). Hannon et al., (Hannon et al., 2015) quantified DNA methylation using Illumina 450K array in normal human post-mortem brain, and reported that DNA methylation levels in cortical regions differ from the cerebellum. Moreover, epigenetic regulation of gene expression is very important in the human brain (Gräff et al., 2011; Numata et al., 2012). However, the extent to which DNA methylation or other epigenetic modifications affect the expression of genes important for human brain function, such as *DAO*/*DAOA*, is largely unknown.

In the present study, our main aim was to understand concomitant DAO and DAOA mRNA and protein expression in the normal human post-mortem brain during development and aging in six brain regions. We also correlated *DAO* mRNA and DAO protein as well as DAO protein and DAOA protein to understand their concomitant expression in different brain regions. Furthermore, we assessed the potential effect of *DAO* and *DAOA* polymorphisms on DAO

and DAOA expression levels to elucidate their role in the regulation of DAO and DAOA expression. To understand the hypothesized regulation of DAO and DAOA expression at the transcription level in an indirect manner, we determined the DNA methylation levels at CpG sites of *DAO* and *DAOA* genes in the cerebellum and the frontal cortex of control human post-mortem brain obtained from Gene Expression Omnibus (GEO) datasets.

5.1.3 Materials and methods

Subjects

Human post-mortem brain samples from six brain regions were studied. These brain samples were from 55 subjects with age ranging from 16 weeks of gestation to 91 years, who had no history of psychiatric or neurological illness, and their post-mortem brain samples showed no neuropathological evidence of any neuropsychiatric disorder. The brain samples were collected from six brain regions, namely: the cerebellum (n=47), brainstem (n=49), amygdala (n=47), striatum (n=52), thalamus (n=52), and frontal cortex (n=54). These brain samples were procured from the Department of Neuropathology, Institute of Pathology, University of Wurzburg, Germany (member of the BrainNet Europe-BNEII) and the London Neurodegenerative Diseases Brain Bank, United Kingdom. Informed written consent for tissue donation was obtained from the individuals or the next of kin. The causes of death of the study population are presented in Supplementary Table S6. This study was approved by the Cantonal Ethic Commission of Zurich (Ref. Nr. EK: KEK-ZH-Nr. 2013-0177).

DNA, RNA and protein isolation

DNA, RNA, and protein were simultaneously isolated from the same frozen human post-mortem brain samples using AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Switzerland). Firstly, frozen brain tissue samples weighing around 27 34 mg were disrupted and homogenized in the buffer provided in the kit using the TissueLyser II (Qiagen, Switzerland). Secondly, tissue lysates were added to the AllPrep DNA spin column, and DNA was eluted. Thirdly, RNA was eluted after adding an ethanol treated sample to a RNeasy spin column. Finally, proteins in the sample were precipitated and pelleted by centrifugation. Since the buffer provided in the kit interferes with protein determination and does not dissolve proteins completely, protein eluates were acetone precipitated, and precipitated proteins were redissolved in urea buffer (7M urea, 2M thiourea, 2% CHAPS and trace bromophenol, pH 8).

TaqMan single nucleotide polymorphism (SNP) genotyping

DNA isolated from post-mortem human cerebellum (n=46), striatum (n=2), thalamus (n=5), and amygdala (n=2) were used for genotyping. DNA concentrations, A260/A280, and A260/A230 ratios were measured using a spectrophotometer (NanoVue Plus, GE Healthcare Life Sciences). Real-time PCR was used to genotype *DAO* (rs3918347, rs4623951), and *DAOA* (rs778293, rs3916971, rs746187) polymorphisms. These SNPs were chosen based on previously reported association with schizophrenia (Allen et al., 2008). DNA,

TaqMan® Genotyping Master Mix (Applied Biosystems, USA), and SNP Genotyping Assays (rs778293 assay number: C_8704507_10, rs3916971 assay number: C_27495752_10, rs746187 assay number: C_1925241_10, rs3918347 assay number: C_27937201_10, and rs4623951 assay number: C_32177440_10, all from Applied Biosystems, USA) were combined in a 384-well plate. Real-time PCR was performed in a C1000^{TM}CFX384^{TM} Thermal cycler (Bio-Rad) using TaqMan® SNP Genotyping Assay PCR standard protocol. Genotypes were determined by the allelic discrimination program of Bio-Rad CFX Manager^{TM} Software version 2.1. The Hardy-Weinberg Equilibrium (HWE) p-value and allele frequencies were computed using PLINK software (Purcell et al., 2007), and $p<0.05$ was considered as statistically significant.

Quantification of *DAO* and *DAOA* mRNA levels using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

RNA isolated from post-mortem human cerebellum (n=44), brainstem (n=40), amygdala (n=39), striatum (n=35), thalamus (n=37), and frontal cortex (n=36) were used for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). A spectrophotometer (NanoVue Plus, GE) was used to measure RNA concentrations, A260/A280, and A260/A230 ratios. RNA integrity was analyzed using Experion automated electrophoresis system (Bio-Rad, Switzerland) in a subset of samples. RNA (500 ng) was reverse transcribed using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Switzerland) as per manufacturer's protocol. In a subset of samples, negative controls were prepared with RNA without reverse transcriptase enzyme. qRT-PCR was performed using cDNA, QuantiFast SYBR Green PCR kit (Qiagen, Switzerland), 1 μM forward and reverse DAO primers (Microsynth, Switzerland), and reference genes [β-actin (*ACTB*) (QT01680476), aminolevulinate synthetase (*ALAS1*) (QT00073122), ribosomal protein L13a (*RPL13A*) (QT00089915), alanyl-tRNA synthetase (*AARS*) (QT00054747), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (QT01192646), peptidyl prolyl isomerase A (*PPIA*) (QT00866137), ribosomal RNA (*R18S*) (QT00019936), and X-prolyl aminopeptidase1 (*XPNPEP1*) (QT00051471); all from Qiagen, , Switzerland]. These eight reference genes were selected based on previous reports of their stability in human post-mortem brain (Durrenberger et al., 2012). The DAO primers used in this study have been described by Verrall et al., (Verrall et al., 2007) (Forward primer: CGCAGACGTGATTGTCAACT; Reverse primer: GGATGATGTACGGGGAATTG). *DAO* mRNA levels were normalized to the reference genes. PCR efficiencies were calculated using LinReg PCR program (Ruijter et al., 2009) and mean PCR efficiencies for all studied amplicons were found to be between 88 and 95%. Normalized *DAO*

mRNA levels were quantified by importing qRT-PCR quantification cycle (Cq) values of the gene of interest and reference genes across brain regions and lifespan into qBASE plus software (Biogazelle, Belgium) which utilizes gene-specific amplification efficiencies, and allows normalization with multiple reference genes. The qBASE plus software automatically selects multiple stably expressed reference genes in the samples across brain regions and lifespan by taking into account their stability. The software carries out a normalization of the gene of interest with multiple reference genes, ultimately producing calibrated normalized relative quantities (CNRQ) of the gene of interest which was used to perform statistical analysis (Hellemans et al., 2007). To detect *DAOA* mRNA in human post-mortem brain, qRT-PCR was performed using several different primers for *DAOA* gene described by Benzel et al., (Benzel et al., 2008), Cheng et al., (Cheng et al., 2014), and pre-designed primers [QT00058863 (Qiagen), Hs.PT.58.555086 (IDT), 4331182 (ThermoFisher scientific), qHsaCEP0024792 (BioRad)]. As a positive control, these primers were also tested on the cDNA prepared from human neuroblastoma cells (SK-N-SH and SH-SY5Y) overexpressing DAOA, and these primers detected *DAOA* mRNA in these cells, but not in the human post-mortem brain samples (data not shown). *DAOA* mRNA was still undetectable in human post-mortem brain after increasing the cDNA amounts in qRT-PCR. Furthermore, amplification using QuantiTect Whole Transcriptome Kit (207043, Qiagen) followed by qRT-PCR to detect *DAOA* mRNA levels was not successful. In summary, we were unable to quantify *DAOA* mRNA levels with any of the aforementioned methods in human post-mortem brain.

Quantification of DAO and DAOA protein levels using commercially available enzyme-linked immunosorbent assay (ELISA) kits

Protein isolated and acetone-precipitated from post-mortem human cerebellum (n=40), brainstem (n=47), amygdala (n=46), striatum (n=48), thalamus (n=48), and frontal cortex (n=54) were used for enzyme-linked immunosorbent assay (ELISA). Total protein concentration was quantified using the Bradford assay (Sigma-Aldrich) (Bradford, 1976). DAO and DAOA protein concentrations were quantified using a commercially available DAO ELISA kit (SEJ298Hu; Cloud-Clone Corp.) with a high specificity and a sensitivity of 0.56 ng/mL and a commercially available DAOA ELISA kit (SEJ297Hu; Cloud-Clone Corp.) with a high specificity and a sensitivity of 0.061 ng/mL according to the manufacturers instructions. A standard curve was obtained by plotting absorbance versus different concentration of protein standards provided with the kit, and DAO and DAOA protein concentrations were determined from

the standard curve. Normalized DAO and DAOA protein concentrations were calculated by dividing DAO and DAOA protein concentrations by the total protein concentration measured using Bradford assay. The specificity of DAO and DAOA ELISA kits was proved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting which is described in detail in supplementary methods S1.

DNA methylation levels across *DAO* and *DAOA* CpG sites in the cerebellum and frontal cortex

The processed and normalized methylation beta values from two datasets: Gene Expression Omnibus (GEO) accession number GSE61431 as described in the study of Pidsley et al.,(Pidsley et al., 2014) and GSE63347 as described by Horvath et al.,(Horvath et al., 2015) were downloaded from the GEO website (http://www.ncbi.nlm.nih.gov/geo/). From these datasets, the methylation beta values for *DAO* and *DAOA* CpG sites from the cerebellum and frontal cortex of controls were extracted. *DAO* (cg03868278, cg22621535, cg22801690) and *DAOA* (cg00809502) CpG sites in the repeat masker region sites were excluded from the analysis. *DAO* CpG sites: cg01694331, cg25362648, cg18037826, cg12592321 lie in the exon 1 and cg26725638 in the exon 2 of *DAO* gene. *DAOA* CpG sites: cg22773522, cg25623522, cg01297020 are in the promoter region, cg13846327 and cg20888753 in the intron 2, and cg11374446 in the intron 3 region of the *DAOA* gene. In the GSE61431 dataset, there were 23 control cerebellum and frontal cortex samples ranging from ages 25 to 96 years. In the GSE63347 dataset, there were 9 control cerebellum samples in the ages between 38 and 64 years and 17 control frontal cortex samples between ages 32 and 64 years.

Statistical analysis

IBM® SPSS® Statistics (version 21) software was used for statistical analysis. Shapiro-Wilk test with Lilliefors significance correction was used to assess the normality of the distribution of mRNA, protein expression, and DNA methylation data. *DAO* mRNA, DAO and DAOA protein expression, and DNA methylation data showed both normal and non-normal distribution across different brain regions. In order to maintain consistency between statistical evaluations, non-parametric tests were used even for normally distributed data. Linear regression with brain regions as dummy variables was used after a logarithmic transformation of the non-normally distributed data to determine the differences in *DAO* mRNA, DAO and DAOA protein expression in the brain regions studied; $p<0.05$ was taken as statistically significant. The differences in *DAO* mRNA, and DAO and DAOA protein levels across different age groups

were assessed by the Kruskal-Wallis test followed by pair-wise comparisons with the Mann-Whitney test by adjusting p-value based on the number of comparisons (Bonferroni correction, $p<0.005$). As we do not have post-mortem brain samples from ages 2 to 20 years, we divided the ages for correlation into two groups, namely less than 2 years and more than 20 years. Then, Spearman's rank correlation test was used to assess the correlation between age groups (<2 years and >20 years) and *DAO* mRNA, DAO and DAOA protein levels; $p<0.05$ was taken as statistically significant. We correlated *DAO* mRNA with DAO protein levels, and DAO protein levels with DAOA protein levels across six brain regions by controlling for age using partial correlation, $p<0.05$ was taken as statistically significant. To analyze the effect of *DAO* and *DAOA* SNP genotypes on *DAO* mRNA, DAO and DAOA protein expression, analysis of covariance (ANCOVA) with age as a covariate was used after a logarithmic transformation of not normally distributed *DAO* mRNA and DAOA protein expression data and the inverse transformation of the non-normally distributed DAO protein expression data; $p<0.05$ was taken as statistically significant. The differences in methylation levels between the cerebellum and frontal cortex at each CpG site was assessed using the Mann-Whitney test by adjusting p-value based on a number of CpG sites analyzed (Bonferroni correction, $p<0.005$). The correlation between age and methylation levels at each CpG site in the cerebellum and frontal cortex was assessed using Spearman's rank correlation test, and $p<0.05$ was considered statistically significant. GraphPad Prism software (version 6.01) was used to plot the graphs. The post-hoc power analyses for *DAO* mRNA, DAO and DAOA protein expression across five age groups were conducted using G*Power software (Faul et al., 2009), the effect sizes were determined from means of age groups, and the alpha level was set at 0.05 (Supplementary Table S7).

5.1.4 Results

DAO and DAOA expression in different brain regions

The current study aimed to assess brain region specific DAO and DAOA expression across the lifespan in normal human post-mortem brain using qRT-PCR and ELISA techniques. The human post-mortem brain samples were obtained from 55 subjects with no clinical or neuropathological evidence of neuropsychiatric disorders. The demographic characteristics of the study sample across five age groups are presented in Supplementary Table S1. In order to assess any confounding factors inherent in human post-mortem studies, we addressed factors such as post-mortem interval (PMI) and gender. The length of PMI did not differ significantly between different age groups as assessed by the Kruskal-Wallis test. Gender had a significant effect on DAO mRNA ($p=0.048$) and protein ($p=0.047$) expression in the thalamus and frontal cortex respectively (Mann-Whitney test, $p<0.05$). *DAO* mRNA in the thalamus and DAO protein in the frontal cortex was significantly more expressed in males than in females. Gender had no significant effect on DAOA protein expression in all brain areas studied. There was no statistically significant correlation between PMI and *DAO* mRNA, DAO and DAOA protein levels in the studied brain regions (Spearman's rank correlation, $p>0.05$).

With the aim of investigating brain region-specific patterns of DAO and DAOA, we assessed their expression in the normal brain. We found that *DAO* mRNA was significantly more expressed in the cerebellum (set to 100%), followed by the brainstem (17.6%), thalamus (4.8%), striatum (2.5%), amygdala (0.9%), and the frontal cortex (0.7%) as assessed by linear regression (Figure 1A and 1B). In contrast to *DAO* mRNA, DAO protein was significantly less expressed in the cerebellum than in the remaining brain regions. In the amygdala (set to 100%), DAO protein was significantly more expressed than in the brainstem (79.2%) (Figure 1C). DAOA protein was significantly more expressed in the frontal cortex (set to 100%) than in the cerebellum (65.2%), amygdala (80.4%), and the thalamus (69.6%). In the striatum (set to 100%), DAOA protein was significantly more expressed than in the amygdala (66.7%) and the cerebellum (54.1%). DAOA protein was significantly less expressed in the thalamus (57.7%) than in the brainstem (85.6%) and striatum (set to 100%) (Figure 1D). Thus, DAO and DAOA exhibit region-specific expression patterns in the human brain.

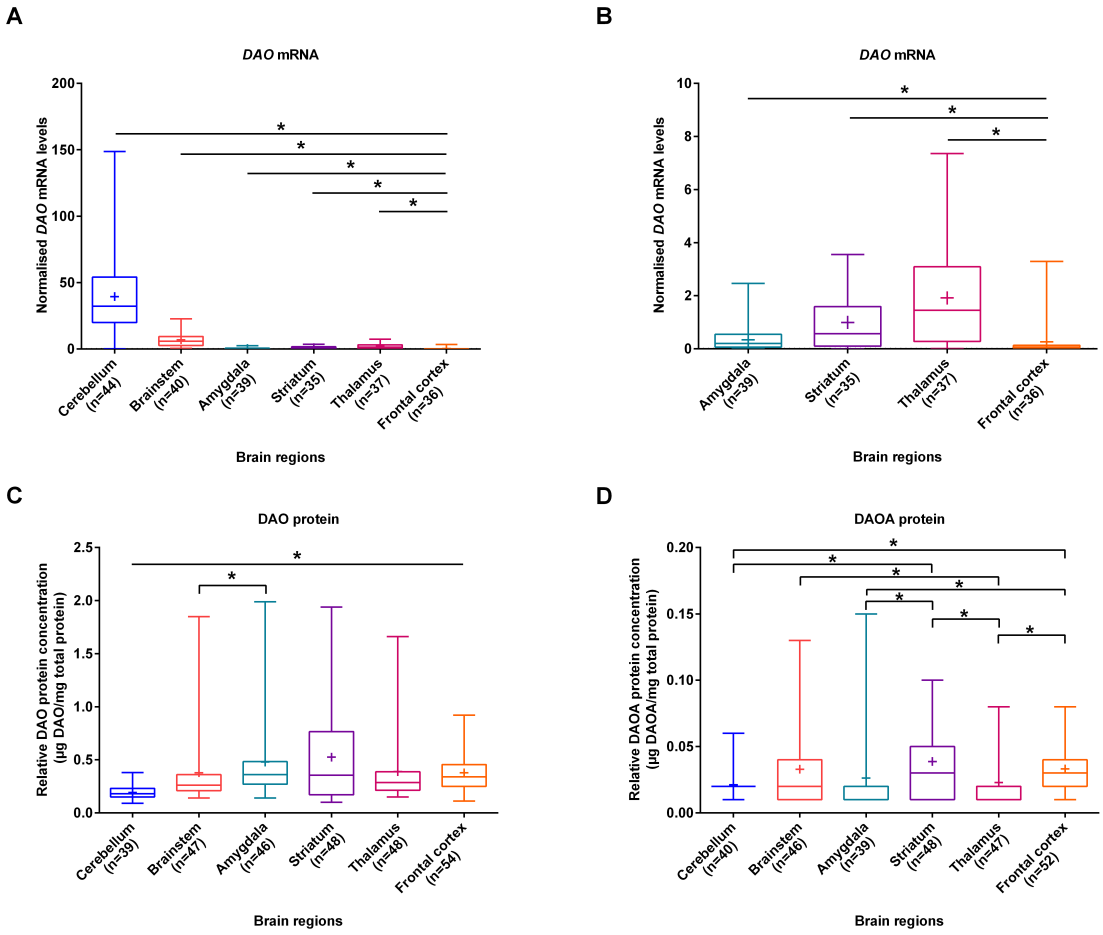


Figure 1: *DAO* mRNA, DAO and DAOA protein levels quantified in six regions of human post-mortem brain. (**A**) Normalized *DAO* mRNA levels across six brain regions. (**B**) For clarity, this figure focuses only on amygdala, striatum, thalamus, and frontal cortex results presented in A. DAO protein levels (μg DAO/mg total protein) (**C**) and DAOA protein levels (μg DAOA/mg total protein) (**D**) in studied brain regions. Values are presented as box and whisker plots, whiskers represent minimum to maximum values, "+" indicates mean values, and *p<0.05. Linear regression with brain regions as dummy variables was used after logarithmic transformation of non-normally distributed data.

Expression of *DAO* mRNA, DAO and DAOA protein across lifespan in six brain regions

The expression patterns across lifespan were analyzed using two approaches. The first approach was by grouping ages into five groups, while the second approach was by correlating ages with expression levels which provided information on expression patterns during brain development, maturation, and degeneration. We assessed DAO and DAOA expression across age groups in six brain regions to understand critical and sensitive periods during brain development. As the sample size in each age group was small, we performed a power analysis which showed reasonable power (>0.76) in the cerebellum, striatum, and thalamus for *DAO* mRNA as well as reasonable power (>0.64)

in the cerebellum, striatum, and frontal cortex for DAO protein, but the power was too low in the brain regions studied for DAOA protein to conduct statistical analysis (Supplementary Table S7).

We found statistically significant ($p<0.05$) differences in *DAO* mRNA levels across the five different age groups, as assessed by the Kruskal-Wallis test, in the cerebellum, brainstem (Figure 2B), amygdala, striatum, and thalamus. However, differences in *DAO* mRNA levels were found to be statistically non-significant in the frontal cortex (Figure 2F). Pair-wise comparisons using the Mann-Whitney test showed statistically significant differences ($p<0.005$) in *DAO* mRNA levels between age groups 0-2 years and >61 years in the cerebellum (Figure 2A) and striatum (Figure 2D), between prenatal and >61 years in the amygdala (Figure 2C), and between prenatal and >61 years, 0-2 years and 36-60 years, 0-2 years and >61 years in the thalamus (Figure 2E).

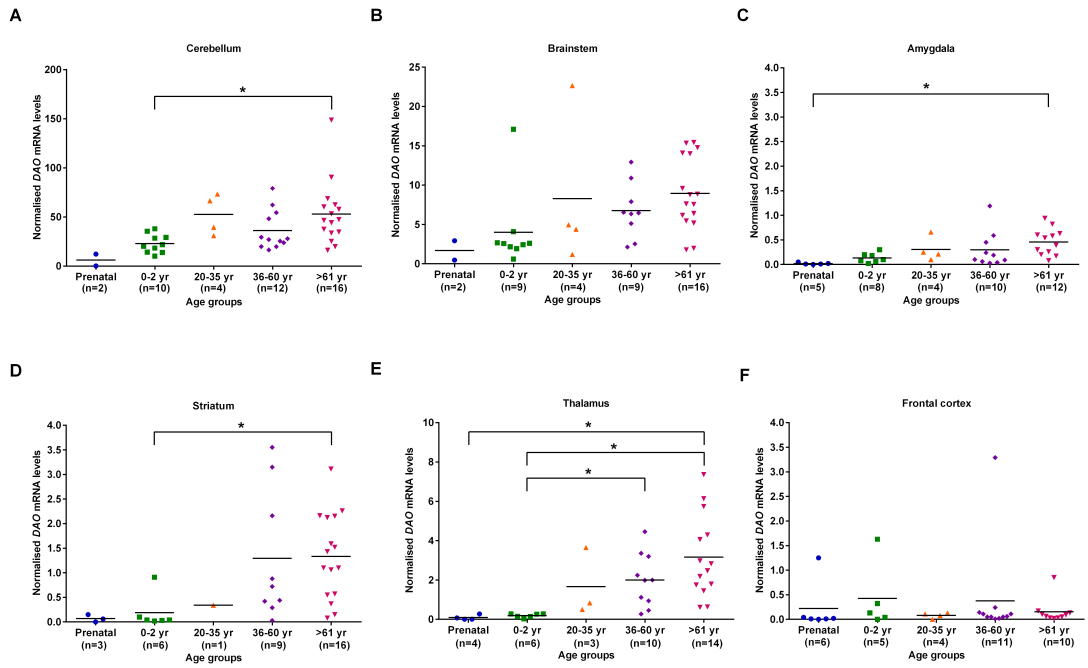


Figure 2: *DAO* mRNA levels across five different age groups in six regions of human post-mortem brain (**A-F**). Data presented as scatter plots. Kruskal-Wallis test followed by pairwise comparisons with the Mann-Whitney test (*$p<0.005$) was performed to assess the differences in *DAO* mRNA levels between different age groups across six brain regions.

The differences in DAO protein levels across the five different age groups was found to be statistically significant ($p<0.05$), as assessed by the Kruskal-Wallis test, in the cerebellum (Figure 3A), striatum (Figure 3D), and frontal cortex (Figure 3F). However, differences in DAO protein levels were found to be statistically non-significant in the brainstem (Figure 3B), amygdala (Figure 3C), and thalamus (Figure 3E). Pair-wise comparisons with the Mann-Whitney test

showed statistically significant differences ($p<0.005$) in DAO protein levels for age groups >61 years versus prenatal and 0-2 years in the frontal cortex (Figure 3F). The differences in DAOA protein levels across the five different age groups were found to be statistically significant ($p<0.05$) in the brainstem as assessed by the Kruskal-Wallis test. There were no statistically significant differences in DAOA protein levels in the cerebellum, amygdala, striatum, thalamus, and frontal cortex (Figure 4). The specificity of DAO and DAOA ELISA kits was demonstrated by Western blot as presented in Supplementary Figure S2.

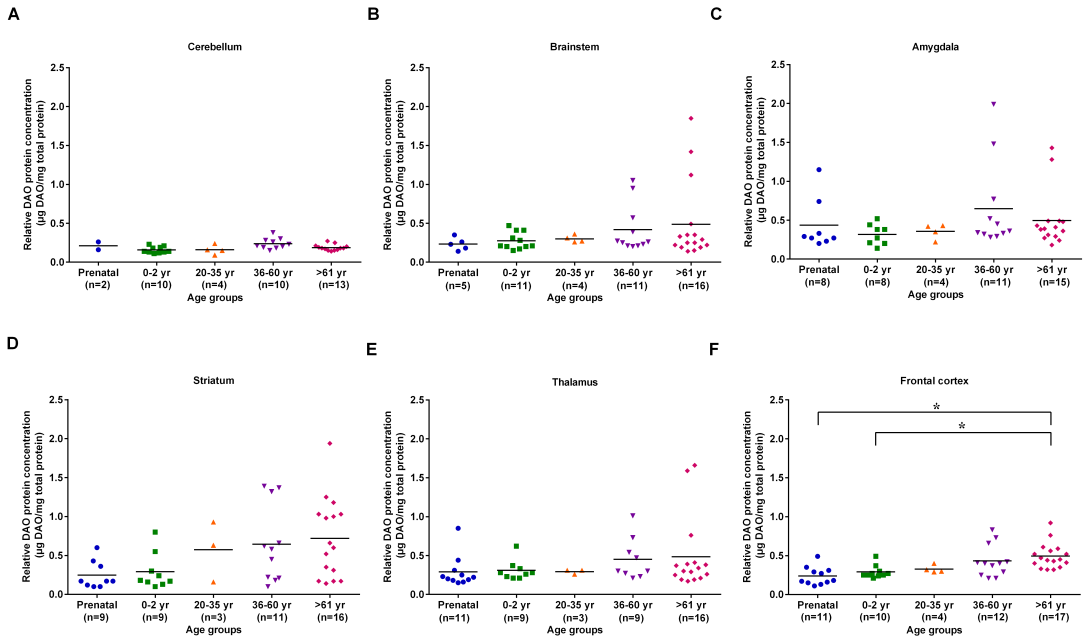


Figure 3: DAO protein levels (μg DAO/mg total protein) across different age groups in six regions of human post-mortem brain (**A-F**). Data presented as scatter plots. Differences in DAO protein levels across different age groups was assessed by the Kruskal-Wallis test followed by pairwise comparisons with the Mann-Whitney test (*$p<0.005$).

Using the second approach, we correlated age (<2 years and >20 years) with DAO and DAOA expression across different brain regions to understand the critical developmental period and brain region in DAO and DAOA expression. We found a statistically significant positive correlation ($p<0.05$) as assessed by Spearman's rank correlation test, between age less than 2 years and *DAO* mRNA levels in the cerebellum and amygdala (Table 1). Furthermore, there was a significant positive correlation (Spearman's rank correlation, $p<0.01$) between age more than 20 years and DAO protein concentration in the frontal cortex (Table 1). There was no statistically significant correlation between age and DAOA protein concentration in the brain regions studied (Table 1).

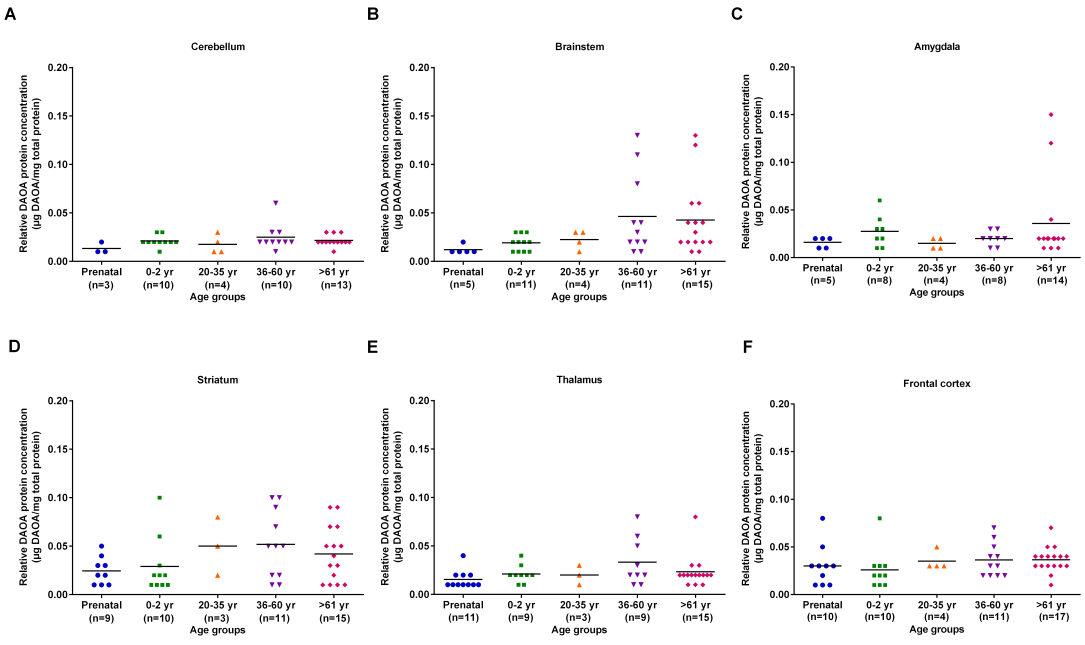


Figure 4: DAOA protein expression (μg DAOA/mg total protein) across five different age groups in six regions of human post-mortem brain (**A-F**). Values are presented as scatter plots. Differences in DAOA protein levels across different age groups were assessed with the Kruskal-Wallis test.

Correlation between *DAO* mRNA, DAO and DAOA protein in different brain regions

In order to assess whether there is a concomitant expression of *DAO* mRNA and its protein, we correlated the two in various brain regions. We did not find any statistically significant correlation (partial correlation controlled for age) between *DAO* mRNA and DAO protein in each of the studied brain regions (Supplementary Figure S3).

As we concomitantly measured DAO and DAOA proteins in the human brain, we examined whether these two proteins correlate in each brain region. A statistically significant positive correlation (partial correlation controlled for age) was found between DAO and DAOA protein expression in the cerebellum [$r(36) = 0.74$, $p < 0.0001$; Figure 5A], the brainstem [$r(43) = 0.84$, $p < 0.0001$; Figure 5B], the amygdala [$r(36) = 0.88$, $p < 0.0001$; Figure 5C], the striatum [$r(43) = 0.84$, $p < 0.0001$; Figure 5D], and the thalamus [$r(44) = 0.91$, $p < 0.0001$; Figure 5E]. However, there was no statistically significant correlation between DAO and DAOA protein concentration in the frontal cortex [$r(49) = 0.27$, $p = 0.06$ Figure 5F].

Table 1: Correlation between age and *DAO* mRNA, DAO and DAOA protein expression across brain regions of human post-mortem brain

	Brain regions	Age in years					
		Prenatal and 0-2 years			20-91 years		
		N	Spearman's rank correlation co-efficient	p-value	N	Spearman's rank correlation co-efficient	p-value
Normalised *DAO* mRNA levels	Cerebellum	12	0.828	**0.001****	32	0.127	0.488
	Brainstem	11	0.310	0.354	29	0.117	0.546
	Amygdala	13	0.669	**0.017***	26	0.261	0.198
	Striatum	9	0.008	0.983	26	0.259	0.202
	Thalamus	10	0.427	0.218	27	0.254	0.200
	Frontal cortex	11	0.352	0.288	25	0.022	0.918
Relative DAO protein concentration (μg DAO/mg total protein)	Cerebellum	12	-0.495	0.102	27	-0.143	0.478
	Brainstem	16	0.092	0.736	31	0.077	0.679
	Amygdala	16	-0.122	0.652	30	0.162	0.392
	Striatum	18	0.082	0.747	30	0.142	0.455
	Thalamus	20	0.285	0.224	28	0.180	0.359
	Frontal cortex	21	0.185	0.422	33	0.482	**0.004***
Relative DAOA protein concentration (μg DAOA/mg total protein)	Cerebellum	13	0.120	0.695	27	0.099	0.623
	Brainstem	16	0.332	0.210	30	0.324	0.081
	Amygdala	13	0.318	0.268	26	0.306	0.121
	Striatum	19	0.037	0.879	29	-0.120	0.536
	Thalamus	20	0.195	0.411	27	0.021	0.916
	Frontal cortex	20	-0.200	0.398	32	0.149	0.417

*$p<0.05$ (**bold font**); **$p<0.01$ (**bold font**)

DAO and DAOA expression across *DAO* and *DAOA* SNP genotypes in different brain regions

We assessed the potential effect of *DAO* and *DAOA* SNPs on DAO and DAOA expression to understand whether such genetic variations known to be risk factors in schizophrenia play a role in the regulation of their expression. The *DAO* and *DAOA* SNPs were in Hardy-Weinberg equilibrium ($p>0.05$), and the minor allele frequency (MAF) of *DAO* and *DAOA* SNPs were similar to HapMap CEU MAF (Supplementary Table S2). In the thalamus, *DAO* mRNA was significantly more expressed in rs3918347 *DAO* genotype GG (minor allele G) than in rs3918347 *DAO* genotypes (GA, AA), and *DAO* mRNA was less expressed in rs3918347 *DAO* genotypes GG+GA than in rs3918347 *DAO* genotype AA as assessed by ANCOVA with age as covariate (Supplementary Table S3). However, for the DAO protein, no statistically significant difference was detected among rs3918347 and rs4623951 *DAO* genotypes (Supplementary Table S4). For DAOA protein expression, there was a significantly higher expression in rs3916971 *DAOA* genotype TT (minor allele T) than in rs3916971 *DAOA* genotypes (CT, CC, CT+CC) in the amygdala, as assessed by ANCOVA with age as a covariate (Supplementary Table S5). Nonetheless, from our findings, we cannot conclusively comment on the effect of *DAO* and *DAOA* SNPs on their expression due to their rather small sample size.

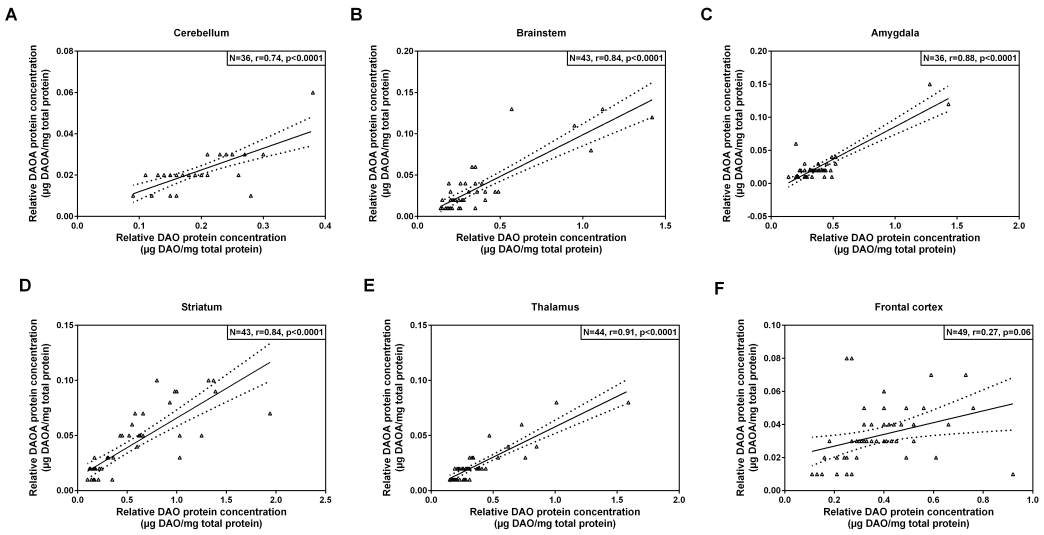


Figure 5: Correlation between DAO (μg DAO/mg total protein) and DAOA protein expression (μg DAOA/mg total protein) in six regions of the human post-mortem brain (**A-F**). Data is presented as scatter plots with linear fit and 95% confidence intervals. Correlation between DAO and DAOA protein levels in different brain regions were assessed with the partial correlation test, controlled for age; $p<0.05$ was taken as statistically significant (N: sample size, r: correlation co-efficient).

DNA methylation across *DAO* and *DAOA* CpG sites in cerebellum and frontal cortex

From our DAO and DAOA mRNA and protein findings, we hypothesized a possible regulation of their expression at the transcriptional levels. To elucidate this, we analyzed *in silico* DNA methylation across *DAO* and *DAOA* CpG sites in the cerebellum and frontal cortex to indirectly explain the differential DAO and DAOA expression in the human brain. In GSE61431 and GSE63347 datasets, gender had no statistically significant effect on DNA methylation levels at *DAO* and *DAOA* CpG sites in the cerebellum and frontal cortex (Mann-Whitney test, $p>0.005$). In GSE61431 and GSE63347 datasets, *DAO* CpG site cg18037826 is significantly more methylated in the cerebellum than in the frontal cortex as assessed by the Mann-Whitney test, $p<0.001$ (supplementary Figure S1A). In the GSE61431 dataset, all studied *DAOA* CpG sites except cg13846327 are statistically significantly ($p<0.001$) more methylated in the cerebellum than in the frontal cortex (Supplementary Figure S1B). In GSE63347 dataset, *DAOA* CpG sites cg22773522, cg20888753, and cg11374446 are significantly more methylated in the cerebellum than in the frontal cortex (Supplementary Figure S1B). There was no statistically significant correlation between age and DNA methylation levels at *DAO* and *DAOA* CpG sites in the cerebellum and frontal cortex in both studied datasets.

5.1.5 Discussion

This study investigated the age-span expression of DAO and DAOA mRNA and protein in six brain regions of normal human post-mortem brain samples. In this study, we also determined *in silico* DNA methylation levels at *DAO* and *DAOA* CpG sites in the cerebellum and frontal cortex of control human post-mortem brain. The results of this study have implications for understanding the regulation and expression of *DAO* and *DAOA* genes in the normal human brain during development and aging.

In this study, DAO mRNA and protein was detected in all brain regions studied. *DAO* mRNA was highly expressed in the cerebellum as compared to the other brain regions studied. This finding is in line with previous studies of DAO expression in the human brain (Kapoor et al., 2006; Verrall et al., 2007; GTEx Consortium, 2015). This is the first study which quantified DAO protein in human post-mortem brain using the quantitative ELISA method. Previous studies detected DAO protein in the human brain using methods such as immunohistochemistry and Western blot (Bendikov et al., 2007; Verrall et al., 2007; Uhlén et al., 2015). Immunohistochemical studies measured DAO immunoreactivity in the human brain and found that DAO protein was robustly detected both in the cerebellum and cerebral cortex (Verrall et al., 2007; Uhlén et al., 2015). Another study detected DAO protein in the frontal cortex and hippocampus using Western blot (Bendikov et al., 2007). We found that DAO protein was less expressed in the cerebellum, but *DAO* mRNA was highly expressed in the cerebellum as compared to other regions studied, which might indicate post-transcriptional regulation such as microRNA (miRNA) mediated regulation. The miRWalk 2.0 database (Dweep and Gretz, 2015) with prediction algorithms from 4 different databases (miRWalk, miRanda, RNA22, Targetscan) showed that there are around 327 predicted miRNA target sites in the promoter region (miRWalk, miRanda, Targetscan), 3 predicted miRNA target sites in the 5'UTR (miRWalk, miRanda, RNA22), 74 predicted miRNA target sites in the coding DNA sequence (CDS) (miRWalk, miRanda, RNA22, Targetscan), and 9 predicted miRNA target sites in the 3'UTR (miRWalk, miRanda, RNA22, Targetscan) of the *DAO* gene. The interaction of these miRNAs with the *DAO* gene in the cerebellum might be the reason for more *DAO* mRNA and less DAO protein. However, these interactions have to be confirmed experimentally. We measured DAO protein but not the activity of DAO protein because the buffer used to isolate proteins deactivated the DAO enzyme. Therefore, in our study, we are unable to comment on the activity of DAO in different brain regions. The DAO protein can be either in an active [Flavin adenine dinucleotide (FAD

bound)] or inactive (FAD unbound) state (Caldinelli et al., 2009; Terry-Lorenzo et al., 2014). Kapoor et al., (Kapoor et al., 2006) showed that DAO activity was detected in the human cerebellum but not in the human forebrain. Moreover, a recent study by Sasabe et al., (Sasabe et al., 2014) showed that DAO activity was detected in the human hindbrain, midbrain, spinal cord, and only in the white matter of the forebrain. Therefore, DAO activity in the human forebrain is not yet very clear.

DAOA is a primate-specific gene which shows a complex alternative splicing pattern, and encodes a protein characterized by a rapidly changing structure during evolution (Chumakov et al., 2002). The DAOA gene and protein expression in the human brain have not yet been fully characterized. We detected DAOA protein using ELISA in all of the brain regions studied. The human protein atlas detected DAOA protein in the cerebellum and cerebral cortex using immunohistochemistry (Uhlén et al., 2015). Kvajo et al., detected DAOA protein in the human amygdala using Western blot (Kvajo et al., 2008). However, Benzel et al., could not detect DAOA protein in the human brain (cerebellum, amygdala, frontal cortex) using Western blot (Benzel et al., 2008). Studies showing *DAOA* mRNA expression in the human brain are limited (Chumakov et al., 2002; Korostishevsky et al., 2006). We were unable to detect *DAOA* mRNA in all the brain regions studied using qRT-PCR, which is in line with previous studies that used methods such as northern blotting, qRT-PCR, and RNA sequencing (Benzel et al., 2008; GTEx Consortium, 2015). The reason for detecting DAOA protein but not *DAOA* mRNA in the human brain might be a tightly regulated DAOA expression or extremely localized expression or post-transcriptional regulation by RNA methylation. A previous study suggested that undetectable *DAOA* mRNA in the human brain might be because of RNA instability motif found in the 5'UTR of *DAOA* gene (Benzel et al., 2008). There are around 314 predicted miRNA target sites in the promoter region (miRWalk, miRanda, Targetscan), 5 predicted miRNA target sites in the 5'UTR (miRWalk, miRanda, RNA22), 317 predicted miRNA target sites in the coding DNA sequence (CDS) (miRWalk, miRanda, Targetscan), and 70 predicted miRNA target sites in the 3'UTR (miRWalk, miRanda, RNA22) of the *DAOA* gene as described in the miRWalk 2.0 database (Dweep and Gretz, 2015) with prediction algorithms from 4 different databases (miRWalk, miRanda, RNA22, Targetscan). The interaction of these miRNAs with the *DAOA* gene might be the reason for the variation of DAOA protein expression in different brain regions. However, the interactions of miRNA with the DAOA gene have to be confirmed experimentally.

During brain development, there are critical and sensitive periods during which the brain is more vulnerable to environmental insults that can potentially lead to psychiatric disorders (Meredith, 2015). In order to understand pathological changes that occur in psychiatric disorders, it is important to analyze the expression of risk genes for psychiatric disorders in the normal brain across the lifespan. In our study, we found that *DAO* mRNA levels were positively correlated with age less than 2 years in the cerebellum and amygdala. Our data is in agreement with previous studies of whole-genome expression analysis in the developing brain which reported that prenatal and neonatal periods are associated with a rapid change of gene expression patterns in the brain as compared to other age periods (Kang et al., 2011; Naumova et al., 2013). During brain development and maturation, synaptic plasticity varies significantly across different brain regions, which might contribute to the brain region-specific gene expression (Huttenlocher and Dabholkar, 1997; Caruana et al., 2012). In this study, DAO expression was brain region specific, indicating that DAO is differentially regulated across hindbrain and forebrain regions. Interestingly, we found a significant positive correlation between DAO and DAOA protein in all the brain regions studied except for the frontal cortex. This positive correlation between DAO and DAOA protein might be because of the variability in density of particular cell types in different brain regions. A recent *in vitro* study showed that there is an interaction between DAO and DAOA proteins (Birolo et al., 2016). Nonetheless, the effect of DAOA protein on DAO is controversial. Chumakov and colleagues (Chumakov et al., 2002) reported DAOA to be an activator of DAO, but Sacchi and colleagues (Sacchi et al., 2008) reported DAOA to be a repressor of DAO. From our finding, we are still unable to conclude regarding DAO and DAOA interaction, but we could show their simultaneous expression.

DNA methylation has been widely acknowledged to be crucial for normal brain development (Fan et al., 2001), and alterations in DNA methylome in the human brain might contribute to neuropsychiatric disorders (Illingworth et al., 2015). DNA methylation can lead to both increases and decreases in gene expression (Wagner et al., 2014). We found *in silico* that the cerebellum was significantly more methylated than the frontal cortex in most of the *DAO* and *DAOA* CpG sites which is in line with previous studies (Davies et al., 2012; Hannon et al., 2015; Illingworth et al., 2015). This finding might explain the differential expression of *DAO* mRNA in the cerebellum and frontal cortex. However, further studies are required to understand the extent to which DNA methylation or other epigenetic modifications affect the expression of genes important for human brain function.

In this study, we attempted to carefully address confounding factors such as PMI and gender that are inherent in human post-mortem studies. Our study is the first study which measured concomitantly DAO and DAOA protein levels in human post-mortem brain using quantitative ELISA method. Nevertheless, we were only able to measure total DAO protein but not the DAO activity in different brain regions because of the isolation procedure used. In our study, we had no post-mortem samples between ages 2 years and 20 years, therefore we are unable to comment on alterations within this particular developmental phase. We also do not have schizophrenia brain samples to comment on regulation and expression of DAO and DAOA mRNA and protein across different brain regions in schizophrenia. *DAO* and *DAOA* are susceptibility genes for schizophrenia and bipolar disorders. Both disorders are discussed as neurodevelopmental disorders, and first symptoms often occur before early adulthood, whereas the definitive onset of the disorder is typically in early adulthood (Cacabelos and Martínez-Bouza, 2011; Rapoport et al., 2012). Therefore, it is important to investigate the regulation of these genes between 2 years and 20 years in the normal human brain. Hence, future studies in this age period would be useful to elucidate this question. Another limitation of the study is that because of limited sample size, it is not powered appropriately, particularly regarding the effect of *DAO* and *DAOA* polymorphisms on their expression. However, the strength of this study lies in the fact that we extracted DNA, RNA and total protein from the same tissue, which allowed us to investigate concomitantly DAO and DAOA expression in six different brain regions and across a wide range of ages.

In summary, our results showed the regional expression of DAO and DAOA with age in normal human post-mortem brain samples. We detected DAOA protein, despite undetectable *DAOA* mRNA levels in the human post-mortem brain.

5.1.6 Supplementary Information

Supplementary Methods

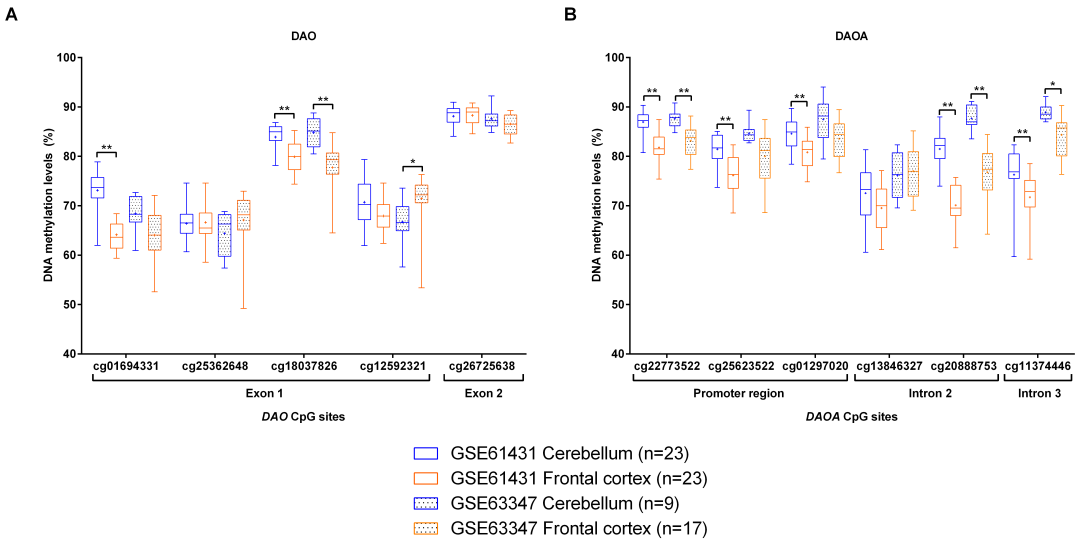
S1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

The acetone precipitated proteins from all six brain regions of subject 081/01 (3 months male) were subjected to electrophoresis on 10-20% Tris-HCl Gel (Bio-Rad) and transferred to PVDF membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk in 1x phosphate buffered saline (PBS) pH 7.4 (Gibco) with 0.1% Tween-20 (Sigma-Aldrich) for 2 hours at room temperature and then it was incubated with primary antibody against DAO (1 μg/ml, same primary antibody that was used in DAO ELISA kit, SEJ298Hu; Cloud-Clone Corp.) or DAOA (1 μg/ml, same primary antibody that was used in DAOA ELISA kit, SEJ297Hu; Cloud-Clone Corp.) at 4°C overnight. The membrane was washed thrice with PBS-T (1x PBS with 0.1% Tween-20) and then incubated with anti-rabbit secondary HRP-conjugated antibody (1:5000, ab97051, abcam) for one hour at room temperature. The protein visualisation was carried out with 20X LumiGLO® Reagent and 20X Peroxide (Cell Signaling) by exposing the membrane to X-ray film in cassette for 1 minute.

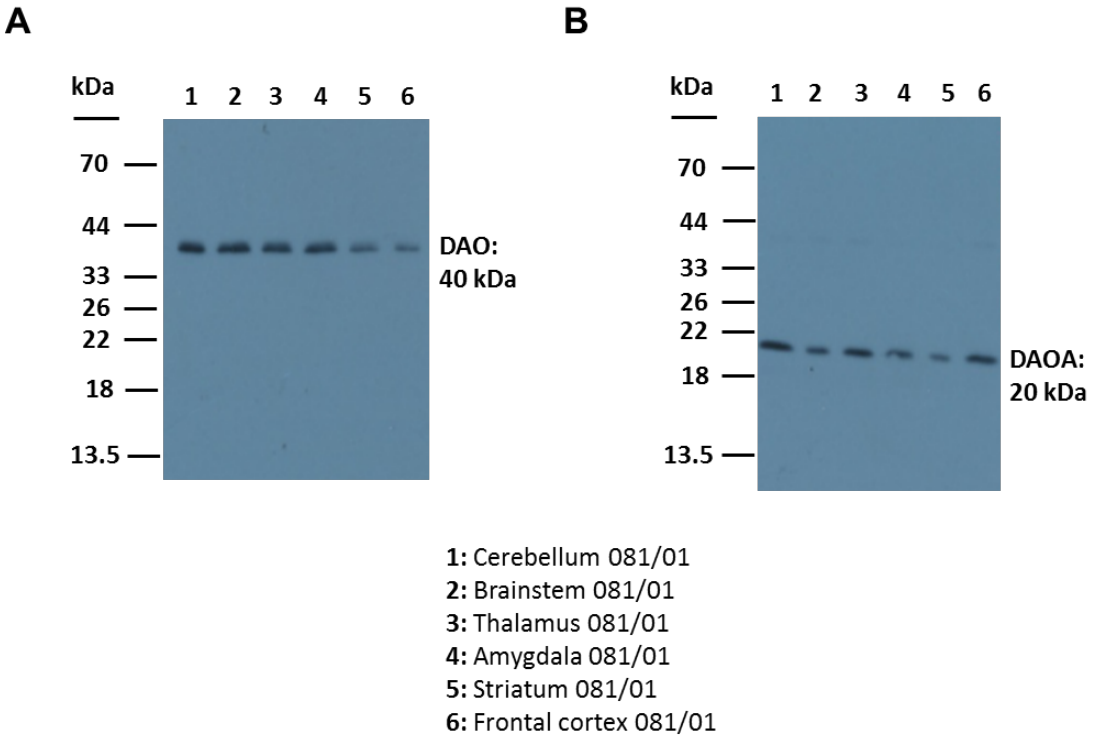
Supplementary Results

To prove the specificity of DAO and DAOA ELISA kits, we performed SDS-PAGE and western blot with acetone precipitated samples of subject 081/01 (3 month old male) from six brain regions. We found that DAO and DAOA proteins were expressed in all six brain regions at the expected sizes of 40 kDa (Supplementary Figure S2A) and 20 kDa (Supplementary Figure S2B) respectively. This finding proves the specificity of the primary antibody used in DAO and DAOA ELISA kits.

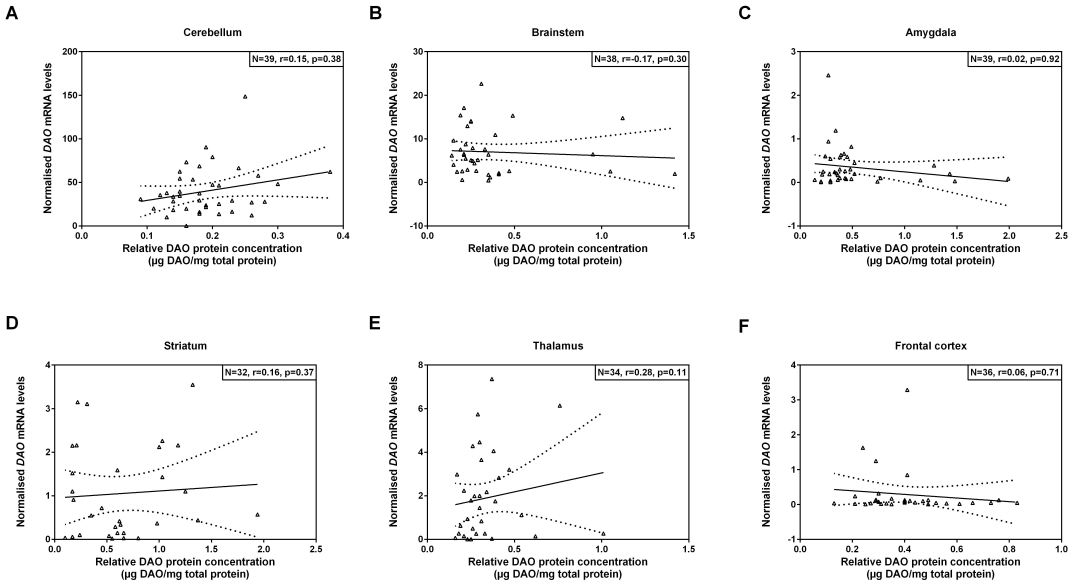
Supplementary Figures



Supplementary Figure S1: Percentage of DNA methylation across *DAO* (**A**) and *DAOA* (**B**) CpG sites in cerebellum and frontal cortex of human brain reported in GEO datasets GSE61431 and GSE63347. Values are presented as box and whisker plots, whiskers represent minimum to maximum values, "+" indicates mean values. Differences in DNA methylation levels between cerebellum and frontal cortex at each CpG site was assessed by the Mann-Whitney test (*p<0.005, **p<0.001).



Supplementary Figure S2: Expression of DAO (**A**) and DAOA (**B**) proteins across six brain regions of subject 081/01. DAO and DAOA proteins are detected in all studied brain regions at the expected sizes of 40 kDa (**A**) and 20 kDa (**B**), respectively.



Supplementary Figure S3: Correlation between *DAO* mRNA and DAO protein expression (μg DAO/mg total protein) in six regions of the human post-mortem brain (**A-F**). Data is presented as scatter plots with linear fit and 95% confidence intervals. Correlation between *DAO* mRNA and DAO protein levels in different brain regions were assessed with the partial correlation test, controlled for age; $p<0.05$ was taken as statistically significant (N: sample size, r: correlation co-efficient).

Supplementary Tables

Supplementary Table S1: Demographic data of the study population by age groups

Age groups	N	Gender		Age (mean±SD)	PMI (mean±SD)
		Male N (%)	Female N (%)		
Prenatal	11	4 (36%)	7 (64%)	21.3±6.9 g.w	36.9±19.9 hours
0-2 years	11	6 (55%)	5 (45%)	0.62±0.60 years	25.5±7.7 hours
20-35 years	4	3 (75%)	1 (25%)	30.5±5.8 years	27.3±20.8 hours
36-60 years	12	6 (50%)	6 (50%)	49.8±6.1 years	26.3±12.1 hours
>61 years	17	11 (65%)	6 (35%)	73.6±8.6 years	25.9±10.2 hours

PMI: post-mortem interval; SD: standard deviation; g.w: gestational weeks

Supplementary Table S2: Genotype distribution and minor allele frequencies of *DAO* and *DAOA* SNPs

Gene	SNP ID	Genotype distribution			Minor allele	MAF	HapMap CEU MAF
		Genotype	N	H-W p-value			
DAO	rs3918347	GG	3	1	G	0.25	0.274
		GA	22				
		AA	31				
	rs4623951	CC	6	0.096	C	0.411	0.412
		CT	34				
		TT	16				
DAOA	rs3916971	TT	13	1	T	0.482	0.394
		CT	28				
		CC	15				
	rs778293	GG	10	0.791	G	0.438	0.389
		AG	29				
		AA	17				
	rs746187	CC	6	0.52	C	0.295	0.375
		CT	21				
		TT	29				

H-W: Hardy-Weinberg; MAF: minor allele frequency; SNP: single nucleotide polymorphism

Supplementary Table S3: Differences in *DAO* mRNA levels among rs3918347 and rs4623951 *DAO* SNP genotypes across six brain regions of human post-mortem brain

***DAO* SNP ID**	**Brain regions**	**Normalised *DAO* mRNA levels**								
		Genotypes (N)	**Mean± SEM**	**p-value[a]**	**Dom model**	**Mean± SEM**	**p-value[a]**	**Rec model**	**Mean± SEM**	**p-value[a]**
rs3918347	Cb	GG (2)	34.7±18.6	0.378	GG+GA	37.1±4.84	0.276	GG	34.7±18.6	0.272
		GA (19)	37.4±5.20					GA+AA	39.6±4.16	
		AA (23)	41.4±6.40		AA	41.4±6.40				
	BS	GG (2)	7.11±1.67	0.366	GG+GA	6.06±0.93	0.17	GG	7.11±1.67	0.506
		GA (16)	5.93±1.03					GA+AA	6.90±0.88	
		AA (22)	7.62±1.33		AA	7.62±1.33				
	Amg	GG (2)	0.39±0.23	0.88	GG+GA	0.25±0.06	0.638	GG	0.39±0.23	0.739
		GA (14)	0.23±0.06					GA+AA	0.33±0.08	
		AA (23)	0.40±0.11		AA	0.40±0.11				
	Str	GG (2)	2.63±0.48	0.728	GG+GA	1.02±0.26	0.636	GG	2.63±0.48	0.61
		GA (16)	0.82±0.24					GA+AA	0.89±0.17	
		AA (17)	0.95±0.23		AA	0.95±0.23				
	TH	GG (2)	3.44±0.62	**0.049***	GG+GA	1.32±0.30	**0.017***	GG	3.44±0.62	0.97
		GA (14)	1.02±0.25					GA+AA	1.82±0.33	
		AA (21)	2.36±0.49		AA	2.36±0.49				
	FC	GG (2)	0.06±0.03	0.313	GG+GA	0.14±0.08	0.126	GG	0.06±0.03	0.537
		GA (13)	0.16±0.09					GA+AA	0.27±0.11	
		AA (21)	0.35±0.17		AA	0.35±0.17				
rs4623951	Cb	CC (6)	55.4±20.7	0.623	CC+CT	36.3±4.71	0.578	CC	55.4±20.7	0.527
		CT (28)	32.2±3.5					CT+TT	36.9±3.35	
		TT (10)	49.9±6.76		TT	49.9±6.76				
	BS	CC (4)	8.15±2.44	0.902	CC+CT	6.63±0.93	0.98	CC	8.15±2.44	0.659
		CT (25)	6.39±1.02					CT+TT	6.78±0.90	
		TT (11)	7.66±1.87		TT	7.66±1.87				
	Amg	CC (5)	0.36±0.17	0.96	CC+CT	0.36±0.09	0.776	CC	0.36±0.17	0.969
		CT (24)	0.36±0.11					CT+TT	0.33±0.08	
		TT (10)	0.28±0.07		TT	0.28±0.07				
	Str	CC (5)	1.26±0.62	0.839	CC+CT	0.88±0.20	0.561	CC	1.26±0.62	0.766
		CT (20)	0.79±0.21					CT+TT	0.94±0.18	
		TT (10)	1.26±0.32		TT	1.26±0.32				
	TH	CC (5)	2.51±0.95	0.662	CC+CT	1.98±0.39	0.403	CC	2.51±0.95	0.556
		CT (20)	1.84±0.44					CT+TT	1.82±0.34	
		TT (12)	1.78±0.55		TT	1.78±0.55				
	FC	CC (6)	0.23±0.13	0.771	CC+CT	0.33±0.14	0.553	CC	0.23±0.13	0.572
		CT (21)	0.35±0.17					CT+TT	0.27±0.12	
		TT (9)	0.07±0.02		TT	0.07±0.02				

[a] p-value by ANCOVA with age as a covariate; *p<0.05 (**bold font**); SEM: standard error of the mean; Dom model: dominant model; Rec model: recessive model; Cb: cerebellum; BS: brainstem; Amg: amygdala; Str: striatum; TH: thalamus; FC: frontal cortex

Supplementary Table S4: Differences in DAO protein levels among rs3918347 and rs4623951 *DAO* SNP genotypes across six brain regions of human post-mortem brain

DAO SNP ID	Brain regions	Relative DAO protein concentration (μg DAO/mg total protein)								
		Genotypes (N)	Mean± SEM	p-value[a]	Dom model	Mean± SEM	p-value[a]	Rec model	Mean± SEM	p-value[a]
rs3918347	Cb	GG (2)	0.18±0.01	0.612	GG+GA	0.19±0.01	0.585	GG	0.18±0.01	0.353
		GA (18)	0.19±0.01					GA+AA	0.19±0.01	
		AA (19)	0.19±0.01		AA	0.19±0.01				
	BS	GG (2)	0.22±0.01	0.428	GG+GA	0.34±0.06	0.563	GG	0.22±0.01	0.205
		GA (20)	0.35±0.07					GA+AA	0.38±0.05	
		AA (25)	0.41±0.08		AA	0.41±0.08				
	Amg	GG (3)	0.34±0.04	0.624	GG+GA	0.44±0.06	0.546	GG	0.34±0.04	0.363
		GA (18)	0.45±0.07					GA+AA	0.49±0.06	
		AA (25)	0.51±0.09		AA	0.51±0.09				
	Str	GG (3)	0.30±0.07	0.637	GG+GA	0.49±0.07	0.507	GG	0.30±0.07	0.631
		GA (20)	0.51±0.08					GA+AA	0.54±0.07	
		AA (25)	0.56±0.10		AA	0.56±0.10				
	TH	GG (3)	0.41±0.02	0.911	GG+GA	0.37±0.07	0.68	GG	0.41±0.02	0.804
		GA (20)	0.36±0.08					GA+AA	0.39±0.05	
		AA (25)	0.41±0.06		AA	0.41±0.06				
	FC	GG (3)	0.37±0.11	0.545	GG+GA	0.38±0.04	0.713	GG	0.37±0.11	0.27
		GA (22)	0.38±0.04					GA+AA	0.38±0.02	
		AA (29)	0.37±0.03		AA	0.37±0.03				
rs4623951	Cb	CC (6)	0.21±0.02	0.632	CC+CT	0.19±0.01	0.392	CC	0.21±0.02	0.499
		CT (24)	0.19±0.01					CT+TT	0.19±0.01	
		TT (9)	0.19±0.01		TT	0.19±0.01				
	BS	CC (6)	0.31±0.07	0.707	CC+CT	0.35±0.05	0.697	CC	0.31±0.07	0.547
		CT (28)	0.36±0.06					CT+TT	0.39±0.06	
		TT (13)	0.45±0.14		TT	0.45±0.14				
	Amg	CC (5)	0.34±0.07	0.498	CC+CT	0.47±0.07	0.795	CC	0.34±0.07	0.289
		CT (29)	0.49±0.08					CT+TT	0.49±0.06	
		TT (12)	0.49±0.09		TT	0.49±0.09				
	Str	CC (5)	0.49±0.16	0.723	CC+CT	0.51±0.07	0.954	CC	0.49±0.16	0.442
		CT (31)	0.51±0.08					CT+TT	0.53±0.07	
		TT (12)	0.58±0.15		TT	0.58±0.15				
	TH	CC (6)	0.45±0.08	0.957	CC+CT	0.38±0.05	0.776	CC	0.45±0.08	0.87
		CT (28)	0.37±0.06					CT+TT	0.38±0.05	
		TT (14)	0.40±0.10		TT	0.40±0.10				
	FC	CC (6)	0.36±0.06	0.892	CC+CT	0.35±0.02	0.895	CC	0.36±0.06	0.679
		CT (33)	0.35±0.03					CT+TT	0.38±0.03	
		TT (15)	0.44±0.06		TT	0.44±0.06				

[a] p-value by ANCOVA with age as a covariate; *p<0.05 (**bold font**); SEM: standard error of the mean; Dom model: dominant model; Rec model: recessive model; Cb: cerebellum; BS: brainstem; Amg: amygdala; Str: striatum; TH: thalamus; FC: frontal cortex

Supplementary Table S5: Differences in DAOA protein levels among rs3916971, rs778293 and rs746187 *DAOA* SNP genotypes across six brain regions of human post-mortem brain

DAOA SNP ID	Brain regions	Relative DAOA protein concentration (μg DAOA/mg total protein)								
		Genotypes (N)	Mean± SEM	p-value[a]	Dom model	Mean± SEM	p-value[a]	Rec model	Mean± SEM	p-value[a]
rs3916971	Cb	TT (6)	0.02±0.002	0.776	TT+CT	0.02±0.002	0.791	TT	0.02±0.002	0.475
		CT (21)	0.02±0.003					CT+CC	0.02±0.002	
		CC (13)	0.02±0.002		CC	0.02±0.002				
	BS	TT (9)	0.05±0.018	0.623	TT+CT	0.03±0.006	0.893	TT	0.05±0.018	0.383
		CT (23)	0.03±0.003					CT+CC	0.03±0.003	
		CC (14)	0.03±0.007		CC	0.03±0.007				
	Amg	TT (7)	0.05±0.021	**0.017***	TT+CT	0.03±0.007	0.719	TT	0.05±0.021	**0.012***
		CT (18)	0.02±0.002					CT+CC	0.02±0.002	
		CC (14)	0.02±0.003		CC	0.02±0.003				
	Str	TT (10)	0.03±0.009	0.922	TT+CT	0.04±0.005	0.935	TT	0.03±0.009	0.686
		CT (26)	0.04±0.006					CT+CC	0.04±0.005	
		CC (12)	0.04±0.009		CC	0.04±0.009				
	TH	TT (10)	0.02±0.004	0.657	TT+CT	0.02±0.003	0.848	TT	0.02±0.004	0.432
		CT (23)	0.03±0.005					CT+CC	0.02±0.003	
		CC (14)	0.02±0.001		CC	0.02±0.001				
	FC	TT (13)	0.03±0.005	0.603	TT+CT	0.03±0.002	0.339	TT	0.03±0.005	0.528
		CT (26)	0.03±0.003					CT+CC	0.03±0.003	
		CC (13)	0.04±0.006		CC	0.04±0.006				
rs778293	Cb	GG (5)	0.02±0.002	0.415	GG+AG	0.02±0.002	0.221	GG	0.02±0.002	0.937
		AG (21)	0.02±0.003					AG+AA	0.02±0.002	
		AA (14)	0.02±0.002		AA	0.02±0.002				
	BS	GG (7)	0.05±0.019	0.241	GG+AG	0.03±0.006	0.123	GG	0.05±0.019	0.237
		AG (23)	0.03±0.005					AG+AA	0.03±0.004	
		AA (16)	0.03±0.007		AA	0.03±0.007				
	Amg	GG (6)	0.04±0.017	0.300	GG+AG	0.03±0.007	0.143	GG	0.04±0.017	0.332
		AG (20)	0.03±0.007					AG+AA	0.02±0.004	
		AA (13)	0.02±0.001		AA	0.02±0.001				
	Str	GG (8)	0.04±0.009	0.538	GG+AG	0.04±0.005	0.637	GG	0.04±0.009	0.400
		AG (26)	0.04±0.006					AG+AA	0.04±0.005	
		AA (14)	0.04±0.008		AA	0.04±0.008				
	TH	GG (9)	0.02±0.004	0.549	GG+AG	0.02±0.003	0.39	GG	0.02±0.004	0.756
		AG (22)	0.02±0.004					AG+AA	0.02±0.003	
		AA (16)	0.02±0.004		AA	0.02±0.004				
	FC	GG (10)	0.03±0.007	0.689	GG+AG	0.03±0.003	0.67	GG	0.03±0.007	0.556
		AG (27)	0.03±0.003					AG+AA	0.03±0.002	
		AA (15)	0.03±0.004		AA	0.03±0.004				
rs746187	Cb	CC (4)	0.03±0.011	0.149	CC+CT	0.02±0.003	0.648	CC	0.03±0.011	0.051
		CT (15)	0.02±0.002					CT+TT	0.02±0.001	
		TT (21)	0.02±0.001		TT	0.02±0.001				
	BS	CC (5)	0.04±0.011	0.679	CC+CT	0.03±0.006	0.764	CC	0.04±0.011	0.376
		CT (18)	0.03±0.006					CT+TT	0.03±0.005	
		TT (23)	0.03±0.007		TT	0.03±0.007				
	Amg	CC (3)	0.01±0.003	0.193	CC+CT	0.03±0.006	0.34	CC	0.01±0.003	0.256
		CT (14)	0.03±0.008					CT+TT	0.03±0.005	
		TT (22)	0.02±0.006		TT	0.02±0.006				
	Str	CC (6)	0.05±0.017	0.631	CC+CT	0.04±0.007	0.354	CC	0.05±0.017	0.546
		CT (18)	0.04±0.007					CT+TT	0.04±0.004	
		TT (24)	0.03±0.005		TT	0.03±0.005				
	TH	CC (5)	0.04±0.012	0.164	CC+CT	0.03±0.004	0.366	CC	0.04±0.012	0.058
		CT (18)	0.02±0.005					CT+TT	0.02±0.002	
		TT (24)	0.02±0.002		TT	0.02±0.002				
	FC	CC (6)	0.03±0.004	0.572	CC+CT	0.03±0.003	0.388	CC	0.03±0.004	0.788
		CT (21)	0.03±0.004					CT+TT	0.03±0.003	
		TT (25)	0.03±0.004		TT	0.03±0.004				

[a] p-value by ANCOVA with age as a covariate; *p<0.05 (**bold font**); SEM: standard error of the mean; Dom model: dominant model; Rec model: recessive model; Cb: cerebellum; BS: brainstem; Amg: amygdala; Str: striatum; TH: thalamus; FC: frontal cortex

Supplementary Table S6: Detailed demographic characteristics of the study population

Sl No.	Gender	Age	PMI (hours)	Cause of death	Brain bank
1	Female	59 yr	24	Pneumonia, cardiac failure	Wurzburg
2	Male	66 yr	19	Cardiovascular failure	Wurzburg
3	Male	65 yr	25	Respiratory insufficiency	Wurzburg
4	Female	0.42 yr	24	Cardiac arrest	Wurzburg
5	Female	84 yr	32	Cardiovascular failure, shock	Wurzburg
6	Male	35 yr	58	Sudden cardiac death	Wurzburg
7	Male	49 yr	14	Haemorrhagic shock, spleen rupture	Wurzburg
8	Female	60 yr	9	Toxic multi-organ failure	Wurzburg
9	Male	73 yr	24	Cardiovascular failure, sepsis	Wurzburg
10	Female	45 yr	41	Sudden cardiac death	Wurzburg
11	Female	41 g.w	7	Aspiration of amniotic fluid, respiratory insufficiency	Wurzburg
12	Female	64 yr	32	Cardiovascular failure, septic shock	Wurzburg
13	Male	0.25 yr	24	Cardiovascular failure	Wurzburg
14	Female	38 yr	20	Aneurism rupture	Wurzburg
15	Male	63 yr	24	Bleeding oesophageal varices	Wurzburg
16	Female	91 yr	12	Circulatory failure	Wurzburg
17	Male	66 yr	23	Acute cardiovascular failure	Wurzburg
18	Female	73 yr	24	Acute myocardial infarction	Wurzburg
19	Female	76 yr	20	Cardiovascular failure	Wurzburg
20	Female	51 yr	12.5	Multi-organ failure	Wurzburg
21	Male	84 yr	48	Acute myocardial infarction	Wurzburg
22	Female	33 yr	18	Multi-organ failure	Wurzburg
23	Male	0.42 yr	48	Respiratory insufficiency	Wurzburg
24	Male	85 yr	20.5	Cardiac arrest	Wurzburg
25	Female	0.125 yr	24	Acute cardiovascular failure	Wurzburg
26	Male	22 yr	21	Cardiovascular failure, sepsis	Wurzburg
27	Female	73 yr	24	Multi-organ failure	Wurzburg
28	Male	46 yr	19	Cardiovascular failure	Wurzburg
29	Male	80 yr	23	Cardiac failure	Wurzburg
30	Male	51 yr	42	Cardiovascular failure	Wurzburg
31	Male	54 yr	27	Respiratory insufficiency	Wurzburg
32	Male	52 yr	44	Myocardial infarction	Wurzburg
33	Male	64 yr	50	Respiratory insufficiency	Wurzburg
34	Male	32 yr	12	Cardiac failure	Wurzburg
35	Male	0.75 yr	24	Cardiovascular failure, septic shock	Wurzburg
36	Male	74 yr	12	Circulatory failure	Wurzburg
37	Male	71 yr	27	Pulmonary embolism	Wurzburg
38	Female	46 yr	27	Cardiac failure	Wurzburg
39	Male	47 yr	36	Cardiac failure after acute myocardial infarction	Wurzburg
40	Female	0.25 yr	17	Accidental asphyxia	London
41	Male	0.375 yr	24	Coronary heart disease	London
42	Male	1.83 yr	24	Coronary heart disease	London
43	Female	1.75 yr	24	Coronary heart disease	London
44	Female	0.29 yr	24	Coronary heart disease	London
45	Male	0.33 yr	24	Coronary heart disease	London
46	Female	20 g.w	36	Spontaneous abortion	London
47	Male	17 g.w	62	Spontaneous abortion	London
48	Male	18 g.w	24	Spontaneous abortion	London
49	Female	18 g.w	14	Unknown	London
50	Female	20 g.w	65	Prostaglandin induction/polydactyly	London
51	Male	19 g.w	65	Unknown	London
52	Female	22 g.w	32	Spontaneous abortion	London
53	Female	23 g.w	42	Unknown	London
54	Male	20 g.w	32	Spontaneous miscarriage	London
55	Female	16 g.w	27	Unknown	London

PMI: post-mortem interval; yr: years; g.w: gestational weeks

Supplementary Table S7: Power analysis for *DAO* mRNA, DAO and DAOA protein expression across age groups

Brain regions	Age groups	*DAO* mRNA		DAO protein		DAOA protein	
		Effect size	Power	Effect size	Power	Effect size	Power
Cerebellum	Prenatal	0.53	0.77	0.55	0.73	0.35	0.34
	0-2 yr						
	20-35 yr						
	36-60 yr						
	>61 yr						
Brainstem	Prenatal	0.42	0.49	0.3	0.3	0.41	0.54
	0-2 yr						
	20-35 yr						
	36-60 yr						
	>61 yr						
Amygdala	Prenatal	0.31	0.26	0.3	0.3	0.31	0.26
	0-2 yr						
	20-35 yr						
	36-60 yr						
	>61 yr						
Striatum	Prenatal	0.57	0.78	0.45	0.65	0.35	0.43
	0-2 yr						
	20-35 yr						
	36-60 yr						
	>61 yr						
Thalamus	Prenatal	0.62	0.82	0.28	0.28	0.36	0.43
	0-2 yr						
	20-35 yr						
	36-60 yr						
	>61 yr						
Frontal cortex	Prenatal	0.19	0.12	0.56	0.9	0.24	0.22
	0-2 yr						
	20-35 yr						
	36-60 yr						
	>61 yr						

yr: years

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5.2 Study II: A systematic meta-analysis of the association of Neuregulin 1 (*NRG1*), D-amino acid oxidase (*DAO*), and DAO activator (*DAOA*)/*G72* polymorphisms with schizophrenia

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Author contributions

VJ performed the literature survey, analysed the data, and wrote the manuscript. MG, CUC, SW, and EG reviewed and revised the manuscript. All authors have approved the final manuscript.

5.2.1 Abstract

The glutamate hypothesis of schizophrenia is related to the proposed dysregulation of D-amino acid oxidase (*DAO*), DAO activator (*DAOA*)/*G72*, and Neuregulin 1 (*NRG1*) genes. Genetic studies have shown significant associations between *DAO*, *DAOA*, *NRG1* single nucleotide polymorphisms (SNPs) and schizophrenia. The systematic literature search yielded 6, 5, and 18 new studies for *DAO*, *DAOA*, and *NRG*1 published after 2011 and not included in the previous SchizophreniaGene (SZGene) meta-analysis. We conducted meta-analyses of 20, 23, and 48 case-control studies, respectively, to comprehensively evaluate the association of 8 *DAO*, 12 *DAOA*, and 14 *NRG1* SNPs with schizophrenia. The updated meta-analyses resulted in the following findings: the C-allele of *DAO* rs4623951 was associated with schizophrenia across all pooled studies [Odds ratio (OR)=0.88, 95% confidence interval (CI)=0.79-0.98, p=0.02, N=3,143)], however, no new reports could be included. The G-allele of *DAOA* rs778293 was associated with schizophrenia in Asian patients (OR=1.17, 95% CI=1.08-1.27, p=0.00008, N=6,117), and the T-allele of *DAOA* rs3916971 was associated with schizophrenia across all studies (OR=0.84, 95% CI=0.73-0.96, p=0.01, N=1,765). Again, for both SNPs, no new eligible studies were available. After adding new reports, the T-allele of *NRG1* SNP8NRG241930 (rs62510682) across all studies (OR=0.95, 95% CI=0.91-0.997, p=0.04, N=22,898) and in Caucasian samples (OR=0.95, 95% CI=0.90-0.99, p=0.03, N=16,014), and the C-allele of *NRG1* rs10503929 across all studies (OR=0.89, 95% CI=0.81-0.97, p=0.01, N=6,844) and in Caucasian samples (OR=0.89, 95% CI=0.81-0.98, p=0.01, N=6,414) were protective against schizophrenia. Our systematic meta-analysis is the most updated one for the association of *DAO*, *DAOA*, and *NRG1* SNPs with schizophrenia.

5.2.2 Introduction

Schizophrenia is a complex heritable disorder characterised by positive symptoms such as delusions, hallucinations, and thought disorder; negative symptoms such as flattening of emotional responses, avolition, apathy, and social withdrawal (American Psychiatric Association, 2013), and cognitive deficits such as impairments in attention, memory, and executive functions (Elvevag and Goldberg, 2000; Fioravanti et al., 2012). Schizophrenia has an estimated heritability of around 60-80% (Pepper and Cardno, 2014). Several pathophysiological hypotheses focusing on dysregulation of neurotransmitter pathways such as dopamine, glutamate, serotonin, and gamma-aminobutyric acid (GABA) have been put forward to explain the pathophysiology of schizophrenia (Eggers, 2013; Brisch et al., 2014; Carlsson et al., 2001; Nakazawa et al., 2012; Veerman et al., 2014). The glutamate hypothesis of schizophrenia is based on a proposed hypofunction of the N-methyl-D-aspartate (NMDA) receptors (Veerman et al., 2014; Hu et al., 2015). One possible explanation for NMDA hypofunction in schizophrenia is increased activity of the enzyme D-amino acid oxidase (DAO) modulated by the D-amino acid oxidase activator (DAOA/G72) leading to decreased D-serine, a co-agonist of the NMDA receptor (Sacchi et al., 2016). Post-mortem studies have shown that there is increased DAO activity in brains of schizophrenia patients compared to the brains of controls (Burnet et al., 2008; Madeira et al., 2008). The effect of DAOA on DAO is controversial, as DAOA has been shown to both increase (Chumakov et al., 2002; Chang et al., 2013) and decrease (Sacchi et al., 2008, 2011) DAO activity. DAOA was also reported to modulate mitochondrial function, thereby playing a role in the pathophysiology of schizophrenia (Kvajo et al., 2008; Otte et al., 2014). Another important gene in the glutamate hypothesis of schizophrenia is Neuregulin 1 (*NRG1*), which is a pleiotropic growth factor involved in normal developmental processes, synaptic plasticity, and neurotransmission (Harrison and Law, 2006). NRG1 exerts its action through binding to its receptor tyrosine kinases, ErbB3 and ErbB4. Altered NRG1/ErbB4 signalling has been described to result in NMDA receptor hypofunction (Li et al., 2007; Mei and Nave, 2014). These studies highlight the potential pathogenic link between dysregulation of *DAO*, *DAOA*, and *NRG1* genes leading to the NMDA receptor hypofunction, which form the basis for the glutamate hypothesis of schizophrenia.

Chumakov and colleagues demonstrated the association of *DAO* and *DAOA/G72* single nucleotide polymorphisms (SNPs) with schizophrenia for the first time (Chumakov et al., 2002). An association of *NRG1* SNPs with schizophrenia was first reported by Stefansson and colleagues (Stefansson

et al., 2002). Subsequently, the SchizophreniaGene (SZGene) meta-analysis (last updated on 23 December 2011) showed a significant association of 24 genetic variants in 16 different genes with schizophrenia (Allen et al., 2008). In that meta-analysis, SNPs of *DAOA* (rs778293, rs3916971) and *NRG1* (rs10503929) were significantly associated with schizophrenia (Allen et al., 2008). In contrast to these findings, a more recent genome-wide association study (GWAS) conducted by the Psychiatric Genomics Consortium (PGC) found that out of 108 schizophrenia associated loci, none were within *NRG1*, *DAO*, and *DAOA* gene regions (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Although there are ambiguous reports on the association of *DAO*, *DAOA*, and *NRG1* SNPs with schizophrenia, these genes still remain candidate genes for schizophrenia because of their role in the glutamatergic signalling that has been associated with schizophrenia in multiple lines of research (Catts et al., 2016; Kantrowitz and Javitt, 2010; Balu and Coyle, 2015; Heresco-Levy et al., 2015; Ledonne et al., 2015). Since the last SZGene meta-analysis update 6 years ago, more than a dozen case-control association studies for *DAO*, *DAOA*, and *NRG1* genes have been published. Thus, we performed an updated systematic review and meta-analysis of both previously meta-analysed and new studies published since 2011 in order to comprehensively evaluate the association of *DAO*, *DAOA*, and *NRG1* SNPs with schizophrenia.

5.2.3 Materials and methods

Search strategy and study selection

A systematic literature search was conducted to include studies that examined associations of *DAO*, *DAOA*, and *NRG1* polymorphisms with schizophrenia. We searched PubMed and Web of Science databases for articles published until March 21, 2017. DAO literature was searched using the keywords: (DAO OR DAAO) AND schizophrenia; DAOA literature was searched using the keywords: (DAOA OR G72) AND schizophrenia, and NRG1 literature was searched using the keywords: (NRG1 OR Neuregulin-1 OR Neuregulin 1 OR Neuregulin1) AND schizophrenia. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram (Liberati et al., 2009) was used to report the search flow for this meta-analysis. Studies included in our meta-analysis had to fulfil the following criteria: (i) detailed description of the sample size, ancestry of participants, and diagnostic criteria for schizophrenia, (ii) case-control studies examining the association between *DAO*, *DAOA*, *NRG1* polymorphisms and schizophrenia, (iii) containing data on allele/genotype frequencies in case and control groups and/or odds ratio (OR) and 95% confidence interval (CI) for the OR, (iv) samples not duplicative of other studies, and (v) at least 3 studies reporting results for each SNP to conduct a meta-analysis. In addition to performing a meta-analysis of all studies pooled together, we conducted a subgroup analysis by dividing the studies into those including patients with Caucasian or Asian ancestry to examine the effects of ethnic heterogeneity. If articles only reported allele/genotype frequencies, OR and 95% CI was calculated from allele frequencies using an online OR calculator (https://www.medcalc.org/calc/odds_ratio.php).

Data synthesis and statistical analysis

The meta-analysis was conducted using the MIX 2.0-Professional software for meta-analysis in Excel, version 2.0.1.4 (http://www.meta-analysis-made-easy.com/) (Bax et al., 2006). The OR of each study was converted to the natural logarithm of OR (Ln (OR)), and 95% CI to standard error (SE) using MIX 2.0 software. The Ln (OR), SE, and sample size (N) were used in the software to perform heterogeneity statistics, heterogeneity funnel plots, and synthesis forest plots. The heterogeneity between studies was assessed by Cochran's chi-square-based Q statistic and the inconsistency index (I^2), with $p<0.05$ being considered statistically significant. If there was significant ($p<0.05$) heterogeneity between studies, we used the random-effects model, otherwise we used the fixed-effects model. The random-effects model considers both between-study and within-study variation, whereas the fixed-effects model considers only

within-study variation (Borenstein et al., 2010). The heterogeneity funnel plots for each included *DAO*, *DAOA*, and *NRG1* SNPs were plotted to visualise the heterogeneity between studies. Synthesis forest plots showing N, OR, 95% CI of each included studies of *DAO*, *DAOA*, and *NRG1* SNPs were created to visualise the association of polymorphisms with schizophrenia in an allelic model, $p<0.05$ being considered statistically significant without Bonferroni correction, and $p<0.0015$ being considered statistically significant with Bonferroni correction. Potential publication bias was assessed using Begg's test (Begg and Mazumdar, 1994) and Egger's regression test (Egger et al., 1997), with $p<0.05$ considered statistically significant. The a-priori and post hoc power analyses for *DAO* (Supplementary Table S11), *DAOA* (Supplementary Table S12), and *NRG1* (Supplementary Table S13) meta-analysis were conducted using G*Power software (Faul et al., 2009), the proportions of cases and controls carrying minor allele were calculated from all studies, z-test was used to measure the difference between these two proportions, and the alpha level was set at 0.05, and for a-priori power analyses, the power was set at 0.8.

5.2.4 Results

DAO meta-analysis

The literature search for studies reporting on the association of *DAO* SNPs with schizophrenia identified 201 non-duplicated articles (Supplementary Figure S1). Out of 201 articles, 170 articles were excluded at the title/abstract level. Altogether, 31 articles were read fully, and 11 articles were excluded because of missing allele/genotype frequencies and/or OR and 95% CI or other reasons (Supplementary Table S1). Finally, 20 studies were included in the meta-analysis (Supplementary Table S2), 6 of which were new studies (Papagni et al., 2011; Mechelli et al., 2012; Sacchetti et al., 2013; Kartalci and Acar, 2016; Liu et al., 2016; Chu et al., 2017), that had not been included in the SZGene meta-analysis. There were at least 3 studies reporting results for 8 *DAO* SNPs: rs4623951, rs2111902, rs3918346, rs3918347, rs3741775, rs3825251, rs2070586, and rs2070587. The meta-analyses summary of all 8 *DAO* SNPs is provided in Table 1. We found a significant association between *DAO* SNP rs4623951 and schizophrenia across all studies, and the minor C-allele was protective against schizophrenia (OR=0.88, 95% CI=0.79-0.98, p=0.02, N=3,143) (Figure 1A). However, no significant association was found between any other *DAO* SNPs (rs2111902, rs3918346, rs3918347, rs3741775, rs3825251, rs2070586, and rs2070587) and schizophrenia across all studies, or in the Caucasian and Asian subsamples (Supplementary Figure S2). The majority of the analyses showed significant between-study heterogeneity ($p<0.05$; Supplementary Figure S3), resulting in the use of random-effects models in these instances. No publication bias was observed ($p>0.05$) in any of the *DAO* meta-analyses as assessed by Begg's test and Egger's regression test (Supplementary Table S3).

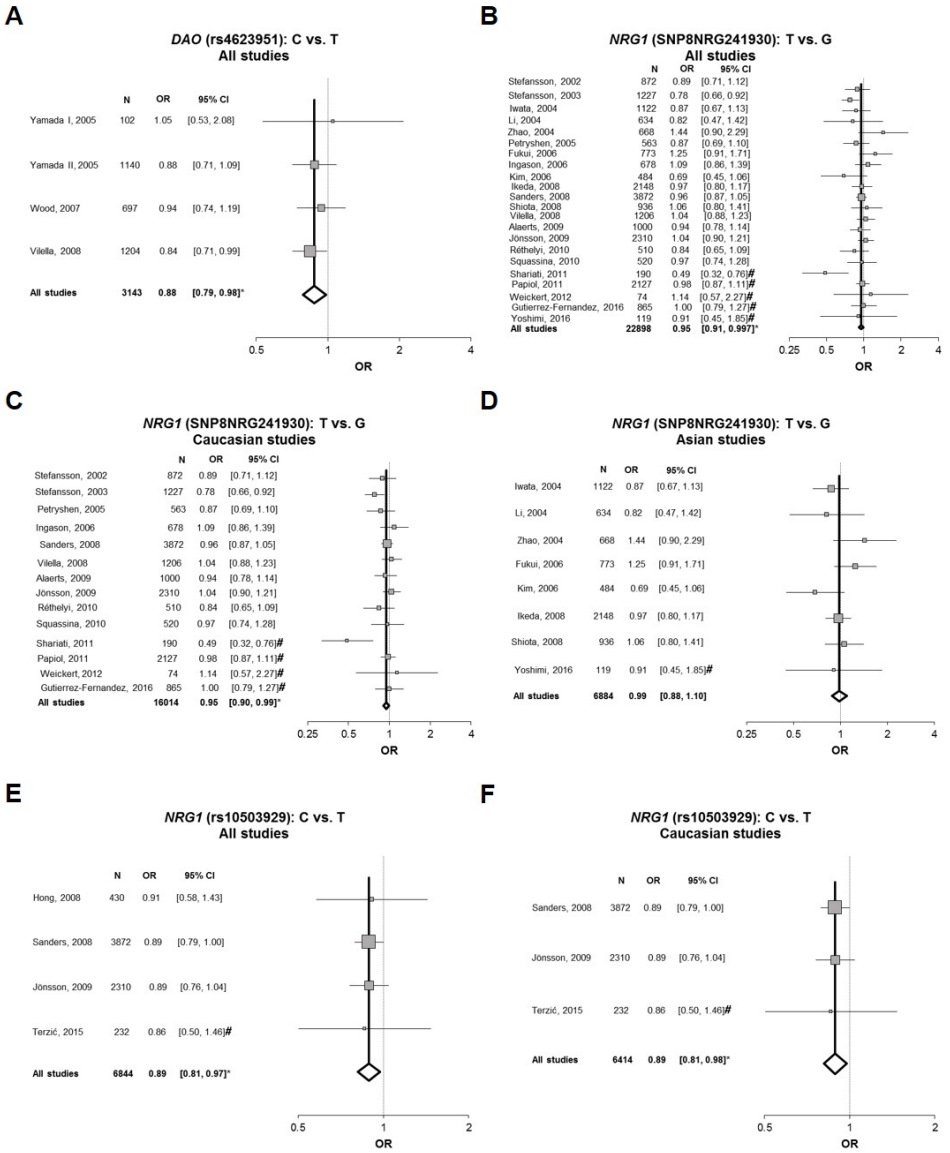


Figure 1: Forest plots of the association between *DAO* rs4623951, *NRG1* SNP8NRG241930 (rs62510682), and *NRG1* rs10503929 polymorphisms, and schizophrenia in the allele model. (**A**) Forest plot of all studies showing association between *DAO* rs4623951 polymorphism and schizophrenia; *p=0.02 (significant without Bonferroni correction). In all studies, C-allele of *DAO* rs4623951 was found to be a protective allele for schizophrenia. (**B**) Forest plot of all studies showing association between *NRG1* SNP8NRG241930 polymorphism and schizophrenia; *p=0.04 (significant without Bonferroni correction). (**C**) Forest plot of only Caucasian studies showing association between *NRG1* SNP8NRG241930 polymorphism and schizophrenia; *p=0.03 (significant without Bonferroni correction). In all studies and Caucasian studies, T-allele of *NRG1* SNP8NRG241930 was found to be a protective allele for schizophrenia. (**D**) Forest plot of only Asian studies showing association between *NRG1* SNP8NRG241930 polymorphism and schizophrenia; p=0.82. (**E**) Forest plot of all studies showing association between *NRG1* rs10503929 polymorphism and schizophrenia; *p=0.01 (significant without Bonferroni correction). (**F**) Forest plot of only Caucasian studies showing association between *NRG1* rs10503929 polymorphism and schizophrenia; *p=0.01 (significant without Bonferroni correction). In all and only Caucasian studies, C-allele of *NRG1* rs10503929 was found to be a protective allele for schizophrenia. In the forest plot, square represents OR of the study, size of the square represents the weight given to each study, whiskers represent 95% CI, and diamond represents the OR and 95% CI of all studies. The new studies published after SZgene meta-analysis are marked as #. Abbreviations: OR: Odds ratio; CI: confidence interval for the OR; DAO: D-amino acid oxidase; NRG1: Neuregulin 1; Minor allele vs. Major allele.

Table 1: Summary of *DAO* polymorphisms meta-analyses

DAO SNP ID	Minor allele	All studies							Caucasian studies							Asian studies						
		Meta-analysis					Heterogeneity		Meta-analysis					Heterogeneity		Meta-analysis					Heterogeneity	
		N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value	N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value	N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value
rs4623951	C	3143	4 (0)	0.88	0.79-0.98	**0.02***	0	0.83	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2111902	G	9598	14 (3)	1.02	0.91-1.14	0.74	65	<**0.001***	6397	9 (2)	1.02	0.86-1.20	0.61	75	<**0.001***	3005	4 (0)	1.04	0.94-1.15	0.43	11	0.34
rs3918346	T	8524	12 (1)	1.00	0.87-1.16	0.97	74	<**0.001***	5519	8 (1)	1.03	0.82-1.30	0.55	81	<**0.001***	3005	4 (0)	0.99	0.90-1.10	0.90	37	0.19
rs3741775	G	11151	14 (3)	1.01	0.89-1.14	0.93	75	<**0.001***	7435	8 (1)	1.01	0.89-1.15	0.82	67	**0.004***	3520	5 (1)	0.99	0.73-1.34	0.95	86	<**0.001***
rs3918347	G	3134	5 (1)	1.11	0.95-1.30	0.18	65	**0.02***	1669	3 (1)	1.20	0.90-1.60	0.21	71	**0.03***	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3825251	G	7298	7 (0)	1.02	0.96-1.09	0.50	37	0.15	4293	3 (0)	1.06	0.80-1.40	0.69	77	**0.01***	3005	4 (0)	1.02	0.95-1.10	0.53	0	0.87
rs2070586	A	5839	6 (2)	1.04	0.89-1.21	0.65	65	**0.01***	2862	3 (1)	1.08	0.74-1.57	0.71	85	**0.001***	2977	3 (1)	1.03	0.92-1.15	0.57	0	0.78
rs2070587	G	7773	6 (2)	1.00	0.93-1.08	0.99	41	0.13	4393	3 (1)	1.03	0.93-1.15	0.56	0	0.76	3380	3 (1)	0.97	0.79-1.18	0.74	73	**0.03***

* $p<0.05$ (**bold font**; significant without Bonferroni correction); #$p<0.0015$ (**bold font** and *italics*; significant with Bonferroni correction); N: total number of cases and controls from all studies; n (new): total number of studies included in the meta-analysis (new studies since SZGene meta-analysis); I^2: inconsistency index; Heterogeneity p-value if <0.05 (**bold font**) random-effects model and if $p>0.05$ fixed-effects model was used; OR: Odds ratio; CI: confidence interval for the OR; SNP: single nucleotide polymorphism; N/A: not available as less than 3 studies available for meta-analysis

DAOA meta-analysis

The literature search studies reporting on the association of *DAOA* SNPs with schizophrenia identified 267 non-duplicated articles (Supplementary Figure S4). Out of 267 articles, 203 articles were excluded at the title/abstract level. Altogether, 64 articles were read fully, and 41 articles were excluded because of missing information on allele/genotype frequencies and/or OR and 95% CI or other reasons (Supplementary Table S4). Finally, 23 articles were included in the meta-analysis (Supplementary Table S5), 5 of which were new studies (Mechelli et al., 2012; Sacchetti et al., 2013; Kartalci and Acar, 2016; Chu et al., 2017; Chen et al., 2013), that had not been included in the SZGene meta-analysis. There were at least 3 studies reporting results for 12 *DAOA* SNPs: rs3916965 (M12), rs3916966 (M13), rs3916967 (M14), rs2391191 (M15), rs947267 (M18), rs778294 (M19), rs3916970 (M20), rs3916971 (M21), rs778293 (M22), rs3918342 (M23), rs1421292 (M24), and rs9558562. The summary of all 12 *DAOA* SNPs meta-analyses is provided in Table 2. There was a significant association between *DAOA* rs778293 (M22) and schizophrenia, and the G-allele was a risk allele for schizophrenia in Asian populations (OR=1.17, 95% CI=1.08-1.27, p=0.00008, N=6,117) (Figure 2C), but not across all studies (Figure 2A) or in Caucasian samples (Figure 2B). We also found a significant association between *DAOA* rs3916971 (M21) and schizophrenia, in which the T-allele was protective across all studies (OR=0.84, 95% CI=0.73-0.96, p=0.01, N=1,765) (Figure 2D), but not in Caucasian populations (Figure 2E). However, there was no significant association between *DAOA* SNPs (rs3916965 (M12), rs3916966 (M13), rs3916967 (M14), rs2391191 (M15), rs947267 (M18), rs778294 (M19), rs3916970 (M20), rs3918342 (M23), rs1421292 (M24), and rs9558562) and schizophrenia (Supplementary Figure S5). The majority of the analyses showed significant between-study heterogeneity ($p<0.05$; Supplementary Figure S6), resulting in the use of random-effects models in these instances. No publication bias was observed ($p>0.05$) in any of the *DAOA* meta-analyses as assessed by Begg's test and Egger's regression test (Supplementary Table S6).

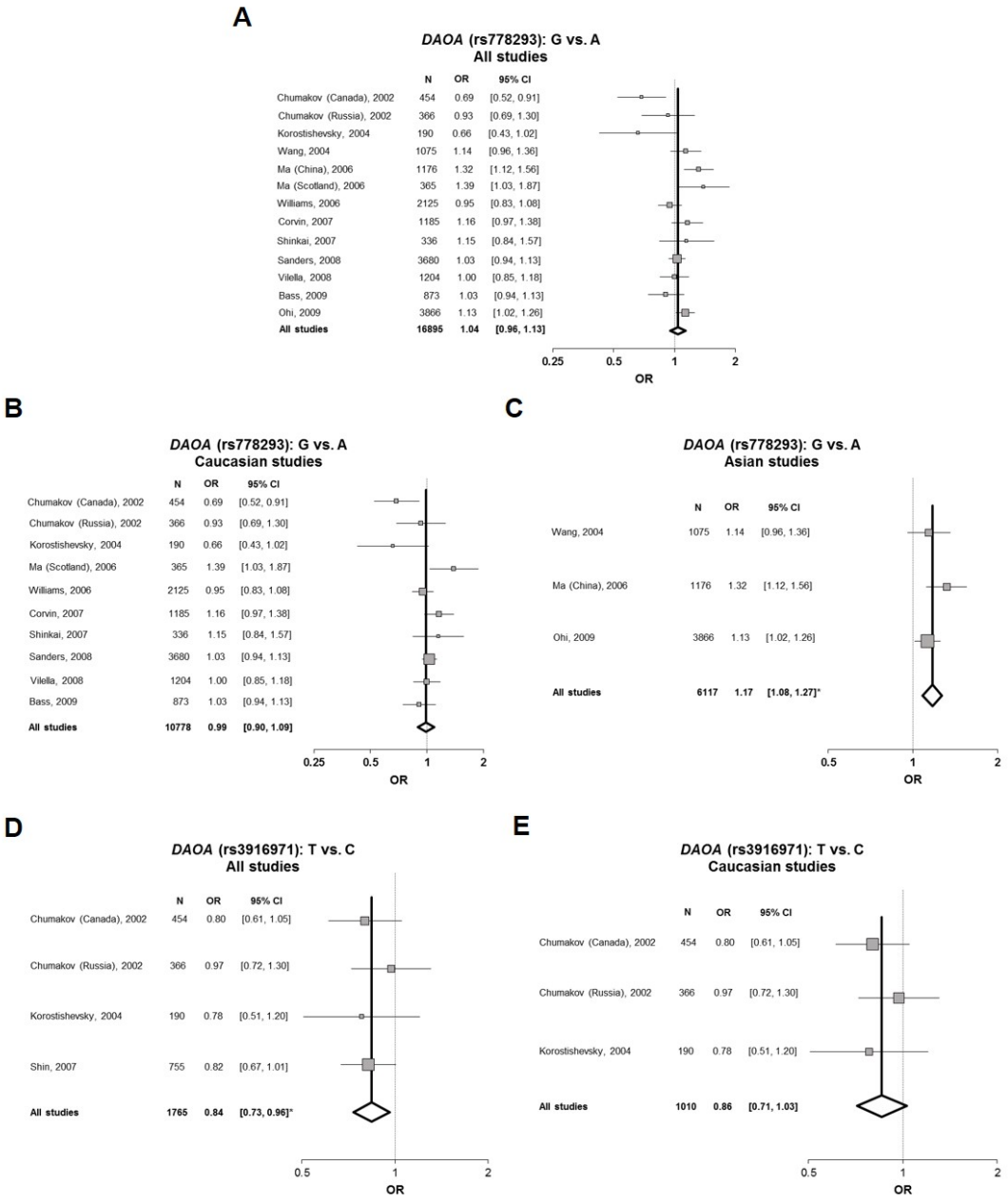


Figure 2: Forest plots of the association between *DAOA* rs778293 (M22) and rs3916971 (M21) polymorphisms, and schizophrenia in the allele model. (**A**) Forest plot of all studies showing association between *DAOA* rs778293 (M22) polymorphism and schizophrenia; p=0.35. (**B**) Forest plot of only Caucasian studies showing association between *DAOA* rs778293 (M22) polymorphism and schizophrenia; p=0.80. (**C**) Forest plot of only Asian studies showing association between *DAOA* rs778293 (M22) polymorphism and schizophrenia; *p=0.00008 (significant with Bonferroni correction). In Asian studies, G-allele of *DAOA* rs778293 (M22) was found to be a risk allele for schizophrenia. (**D**) Forest plot of all studies showing association between *DAOA* rs3916971 (M21) polymorphism and schizophrenia; *p=0.01 (significant without Bonferroni correction). In all studies, T-allele of *DAOA* rs3916971 (M21) was found to be a protective allele for schizophrenia. (**E**) Forest plot of only Caucasian studies showing association between *DAOA* rs3916971 (M21) polymorphism and schizophrenia; p=0.09. In the forest plot, square represents OR of the study, size of the square represents the weight given to each study, whiskers represent 95% CI, and diamond represents the OR and 95% CI of all studies. The new studies published after SZgene meta-analysis are marked as #. Abbreviations: OR: odds ratio; CI: confidence interval for the OR; DAOA: D-amino acid oxidase activator; Minor allele vs. Major allele.

Table 2: Summary of *DAOA* polymorphisms meta-analyses

DAOA SNP ID	Minor allele	All studies							Caucasian studies							Asian studies						
		Meta-analysis					Heterogeneity		Meta-analysis					Heterogeneity		Meta-analysis					Heterogeneity	
		N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value	N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value	N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value
rs3916965 (M12)	A	18494	16 (2)	0.98	0.91-1.06	0.67	58	**0.001***	10173	10 (0)	0.95	0.85-1.07	0.42	67	**0.001***	8321	6 (2)	1.03	0.96-1.11	0.38	3	0.40
rs3916966 (M13)	C	6379	6 (1)	0.95	0.89-1.02	0.17	0	0.55	4690	4 (0)	0.95	0.88-1.03	0.25	23	0.27	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3916967 (M14)	G	16049	14 (1)	0.999	0.95-1.05	0.96	23	0.21	8243	9 (0)	0.995	0.94-1.05	0.86	51	**0.04***	7806	5 (1)	1.01	0.92-1.11	0.83	0	0.96
rs2391191 (M15)	A	24455	22 (3)	1.02	0.95-1.09	0.65	64	<**0.001***	15220	14 (0)	0.99	0.90-1.09	0.79	70	<**0.001***	9039	7 (2)	1.06	0.99-1.13	0.07	46	0.08
rs947267 (M18)	C	6947	11 (2)	1.04	0.92-1.17	0.51	58	**0.009***	4025	7 (0)	1.02	0.93-1.12	0.66	30	0.20	2922	4 (2)	1.03	0.77-1.37	0.84	80	**0.002***
rs778294 (M19)	T	18922	14 (2)	0.97	0.93-1.02	0.3	18	0.26	11473	9 (1)	0.98	0.92-1.04	0.5	0	0.45	7449	5 (1)	0.96	0.81-1.13	0.59	18	0.10
rs3916970 (M20)	A	5631	5 (0)	1.06	0.98-1.14	0.14	0	0.95	1010	3 (0)	1.04	0.86-1.24	0.7	0	0.75	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3916971 (M21)	T	1765	4 (0)	0.84	0.73-0.96	**0.01***	0	0.75	1010	3 (0)	0.86	0.71-1.03	0.09	0	0.58	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs778293 (M22)	G	16895	13 (0)	1.04	0.96-1.13	0.35	64	**0.0007***	10778	10 (0)	0.99	0.90-1.09	0.80	57	**0.01***	6117	3 (0)	1.17	1.08-1.27	***0.00008***#	21	0.28
rs3918342 (M23)	T	20541	19 (2)	1.00	0.92-1.09	0.95	72	<**0.001***	12257	13 (0)	1.00	0.89-1.13	0.97	76	<**0.001***	8284	6 (2)	1.05	0.98-1.11	0.17	76	0.05
rs1421292 (M24)	A	4968	5 (2)	0.95	0.88-1.04	0.3	4	0.39	3519	3 (0)	0.94	0.85-1.03	0.17	6	0.35	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs9558562	G	2326	3 (3)	0.93	0.77-1.13	0.47	0	0.44	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

* p<0.05 (**bold font**; significant without Bonferroni correction); #p<0.0015 (**bold font** and *italics*; significant with Bonferroni correction); N: total number of cases and controls from all studies; n (new): total number of studies included in the meta-analysis (new studies since SZGene meta-analysis); I^2: inconsistency index; Heterogeneity p-value if <0.05 (**bold font**) random-effects model and if p>0.05 fixed-effects model was used; OR: Odds ratio; CI: confidence interval for the OR; SNP: single nucleotide polymorphism; N/A: not available as less than 3 studies available for meta-analysis

NRG1 meta-analysis

The literature search studies reporting on the association of *NRG1* SNPs with schizophrenia identified 853 non-duplicated articles (Supplementary Figure S7). Out of 853 articles, 753 articles were excluded at the title/abstract level. Altogether, 100 articles were read fully, and 52 articles were excluded because of missing information on allele/genotype frequencies and/or OR and 95% CI or other reasons (Supplementary Table S7). Finally, 48 articles were included in the meta-analysis (Supplementary Table S8), 18 of which were new studies (Supplementary Table S8, serial no. 31-48) that had not been included in the SZGene meta-analysis. We identified at least 3 studies reporting results for 14 *NRG1* SNPs: rs10503929, SNP8NRG241930 (rs62510682), rs6994992, SNP8NRG221132 (rs73235619), rs776401, rs35753505, rs6988339, SNP8NRG433E1006 (rs113317778), rs3924999, rs2954041, rs4733376, rs2439272, rs2466058, and rs7014762. The summary of all these 14 *NRG1* SNPs meta-analyses is described in Table 3. We found a significant association between *NRG1* SNP8NRG241930 (rs62510682) and schizophrenia, and the minor T-allele was protective across all studies (OR=0.95, 95% CI=0.91-0.997, p=0.04, N=22,898) (Figure 1B) as well as in Caucasian populations (OR=0.95, 95% CI=0.90-0.99, p=0.03, N=16,014) (Figure 1C), but not in Asian populations (Figure 1D). There was also a significant association between *NRG1* SNP rs10503929 and schizophrenia, and the minor C-allele was protective against schizophrenia across all studies (OR=0.89, 95% CI=0.81-0.97, p=0.01, N=6,844) (Figure 1E) and Caucasian samples (OR=0.89, 95% CI=0.81-0.98, p=0.01, N=6,414) (Figure 1F). There was no significant association between *NRG1* SNPs (rs6994992, SNP8NRG221132 (rs73235619), rs776401, rs35753505, rs6988339, SNP8NRG433E1006 (rs113317778), rs3924999, rs2954041, rs4733376, rs2439272, rs2466058, and rs7014762) and schizophrenia across all studies, and in Caucasian and Asian subsamples (Supplementary Figure S8). The majority of the analyses showed significant between-study heterogeneity ($p<0.05$; Supplementary Figure S9), resulting in the use of random-effects models in these instances. No publication bias was observed ($p>0.05$) in any of the *NRG1* meta-analyses as assessed by Begg's test and Egger's regression test (Supplementary Table S9).

We compared our *DAO*, *DAOA*, and *NRG1* meta-analyses data with the published PGC GWAS data (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). We found that only *NRG1* SNP8NRG241930 (rs62510682) was significantly associated with schizophrenia (minor T-allele was protective) in both PGC GWAS (OR=0.97, p=0.03) and our meta-analysis (OR=0.95, p=0.04; Supplementary Table S10).

We downloaded linkage disequilibrium (LD) plots of *DAO*, *DAOA*, and *NRG1* SNPs across all, European, and Asian populations from LDlink, a web-based application from the National Cancer Institute of National Institutes of Health (NIH) (Machiela and Chanock, 2015). We found *DAO* SNPs, rs2070586 and rs2070587 in European population, and rs3918346 and rs3918346 in Asian population were in strong LD with a R^2 threshold of 0.8 (Supplementary Figure S10). We also found *DAOA* SNPs, rs3916965 and rs3916967, rs3916965 and rs2391191, rs3916967 and rs2391191 in all, the European, and Asian population, rs3916965 and rs3916966, rs3916966 and rs3916967, and rs1421292 and rs3918342 in the European and Asian population to be in strong LD with a R^2 threshold of 0.8 (Supplementary Figure S11). There were no *NRG1* SNPs in strong LD with a R^2 threshold of 0.8 (Supplementary Figure S12).

Table 3: Summary of *NRG1* polymorphisms meta-analyses

NRG1 SNP ID	Minor allele	All studies							Caucasian studies							Asian studies						
		Meta-analysis					Heterogeneity		Meta-analysis					Heterogeneity		Meta-analysis					Heterogeneity	
		N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value	N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value	N	n (new)	OR	95% CI	p-value	I^2 (%)	p-value
SNP8NRG241930 (rs62510682)	T	22898	22 (5)	0.95	0.91-0.997	**0.04***	30	0.09	16014	14 (4)	0.95	0.90-0.99	**0.03***	37	0.08	6884	8 (1)	0.99	0.88-1.10	0.82	23	0.25
rs10503929	C	6844	4 (1)	0.89	0.81-0.97	**0.01***	0	1.00	6414	3 (1)	0.89	0.81-0.98	**0.01***	0	0.99	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs6994992	T	27820	25 (7)	1.02	0.96-1.07	0.56	49	**0.003***	15471	12 (2)	1.04	0.96-1.12	0.39	55	0.01	10126	11 (4)	1.04	0.98-1.10	0.19	41	0.07
rs35753505	C	23925	29 (5)	1.04	0.99-1.10	0.14	39	**0.02***	16506	17 (1)	1.05	0.98-1.13	0.18	52	**0.006***	7419	12 (4)	1.03	0.97-1.10	0.33	9	0.36
rs6988339	G	9612	6 (2)	0.995	0.90-1.10	0.93	57	**0.04***	6173	3 (0)	0.96	0.89-1.03	0.22	62	0.07	9612	3 (2)	1.05	0.95-1.15	0.37	53	0.12
SNP8NRG221132 (rs73235619)	A	10691	8 (1)	0.98	0.90-1.07	0.62	0	0.49	10691	8 (1)	0.98	0.90-1.07	0.62	0	0.49	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3924999	A	15139	13 (3)	0.98	0.93-1.03	0.34	14	0.31	9412	6 (2)	0.996	0.94-1.06	0.89	25	0.25	5297	6 (2)	0.94	0.86-1.03	0.21	13	0.33
SNP8NRG433E1006 (rs113317778)	A	8656	6 (0)	0.98	0.81-1.19	0.87	56	**0.046***	7534	5 (0)	0.99	0.80-1.21	0.89	64	**0.02***	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2954041	T	11282	6 (2)	1.05	0.95-1.16	0.34	0	0.56	7920	3 (0)	1.03	0.80-1.33	0.81	0	0.63	3362	3 (2)	1.05	0.95-1.17	0.35	33	0.22
rs776401	C	8483	4 (0)	0.97	0.85-1.11	0.69	85	**0.02***	8483	4 (0)	0.97	0.85-1.11	0.69	85	**0.02***	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2439272	A	7578	5 (1)	0.82	0.67-1.00	0.06	82	<**0.001***	6173	3 (0)	0.79	0.60-1.04	0.09	89	<**0.001***	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2466058	T	7519	4 (0)	1.08	0.88-1.32	0.47	62	**0.045***	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs4733376	G	10340	5 (1)	1.04	0.92-1.17	0.57	57	**0.049***	6173	3 (0)	1.11	0.87-1.41	0.39	66	**0.049***	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs7014762	A	3260	3 (2)	1.04	0.93-1.18	0.47	52	0.12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

* $p<0.05$ (**bold font**; significant without Bonferroni correction); #$p<0.0015$ (**bold font** and *italics*; significant with Bonferroni correction); N: total number of cases and controls from all studies; n (new): total number of studies included in the meta-analysis (new studies since SZGene meta-analysis); I^2: inconsistency index; Heterogeneity p-value if <0.05 (**bold font**) random-effects model and if $p>0.05$ fixed-effects model was used; OR: Odds ratio; CI: confidence interval for the OR; SNP: single nucleotide polymorphism; N/A: not available as less than 3 studies available for meta-analysis

5.2.5 Discussion

In our comprehensive meta-analysis focussing on *DAO*, *DAOA*, and *NRG1* SNPs implicated in the glutamate hypothesis of schizophrenia, we found significant associations between schizophrenia and *DAO* (rs4623951, protective across all studies), *DAOA* (rs778293, schizophrenia risk in Asian samples; rs3916971, protective across all studies), *NRG1* (rs10503929, protective across all studies and in Caucasian samples; and SNP8NRG241930 (rs62510682), schizophrenia risk across all studies and in Caucasian samples) gene variations. A meta-analysis using only case-control data showed that of the 75 statistically significant genes, *DAOA*, *DAO*, and *NRG1* were fourth, eighth, and sixteenth in the list, respectively (Sun et al., 2008).

The C-allele of *DAO* rs4623951 was protective against schizophrenia across all studies. However, in this particular SNP, we could not include any new association studies to increase the sample size due to the unavailability of required information, which led to the same report as in the SZGene meta-analysis (Allen et al., 2008) and an earlier meta-analysis (Shi et al., 2008). Since the sample size for this particular SNP (N=3,143) is rather small to medium compared to other analysed *DAO* SNPs, there is a clear need for more association studies to enlarge the sample size to rule out a possible type I error and conclude on its association with schizophrenia. *DAO* rs4623951 SNP is located in the 5'-UTR of the *DAO* gene and has been associated with sensorimotor gating, working memory, and personality patterns in healthy males (Roussos et al., 2011). However, the mechanism underlying the association of *DAO* rs4623951 with schizophrenia remains unclear.

In our meta-analysis, we were also only able to include the same number of studies for *DAOA* rs778293 and rs3916971 as in the SZGene meta-analysis (Allen et al., 2008) due to the unavailability of information in newer studies. As already reported by the SZGene meta-analysis (Allen et al., 2008), the G-allele of *DAOA* rs778293 in Asian samples was a risk allele for schizophrenia, whereas the T-allele of *DAOA* rs3916971 across all studies was protective against schizophrenia. Compared to the other meta-analysed *DAOA* SNPs, the sample sizes for *DAOA* rs778293 and rs3916971 were medium (N=6,117) and small (N=1,765), respectively. Thus, there is a need for more association studies regarding these two *DAOA* SNPs before being able to more conclusively comment on their association with schizophrenia. *DAOA* rs778293 and rs3916971 are located in the intergenic region and 5'-UTR of the *DAOA* gene respectively (Grigoroiu-Serbanescu et al., 2010), but the biological mechanism of these SNPs has not yet been identified. A recent meta-analysis failed to find a significant

association between *DAOA* (rs2391191, rs947267, and rs3918342) SNPs and schizophrenia (Tan et al., 2014), which is also in accordance with our findings, despite adding two more recent studies (Kartalci and Acar, 2016; Chu et al., 2017) in our current analysis (Supplementary Figure S6D, S6E, S6H).

The C-allele of *NRG1* rs10503929 was protective against schizophrenia across studies and in Caucasian samples, which is in accordance with the SZGene meta-analysis (Allen et al., 2008). In the current meta-analysis, we were able to add one more study (Terzić et al., 2015) increasing the power of the meta-analysis by 4%. Our finding is also in line with another recent meta-analysis (Mostaid et al., 2017), which included one more study than our meta-analysis and found a significant association of *NRG1* rs10503929 with schizophrenia. Furthermore, we found the T-allele of *NRG1* SNP8NRG241930 (rs62510682) across all studies and in Caucasian samples to be protective against schizophrenia which is in line with a recent meta-analysis that included both case-control and family studies (Mostaid et al., 2017), as well as PGC GWAS (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Contrary to our current study and the Mostaid et al study, *NRG1* SNP8NRG241930 (rs62510682) SNP was not found to be significantly associated with schizophrenia in the SZGene meta-analysis (Allen et al., 2008). In the current meta-analysis, we included five more studies (Shariati et al., 2011; Papiol et al., 2011; Weickert et al., 2012; Gutiérrez-Fernández et al., 2014; Yoshimi et al., 2016) which increased the power of our meta-analysis by 10%. We found only *NRG1* SNP8NRG241930 (rs62510682) SNP to be associated with schizophrenia in both PGC GWAS and our meta-analysis, but not other SNPs studied. This might be because of different inclusion criteria, multiple testing, and heterogeneous samples (e.g., ethnicity and diagnostic criteria) used in PGC GWAS compared to our meta-analysis. A meta-analysis published in 2013 (Loh et al., 2013) showed no association between *NRG1* (rs3924999 and rs2954041) SNPs and schizophrenia. And even in our current meta-analysis, which includes an additional 7 studies (Réthelyi et al., 2010; Kang et al., 2012; Kim et al., 2012; Díez et al., 2014; Ma et al., 2014; Terzić et al., 2015; He et al., 2016), we continue to find no significant association between *NRG1* (rs3924999 and rs2954041) SNPs and schizophrenia (Supplementary Figure S8H-I). In contrast to our findings, the T-allele of *NRG1* rs2954041 was found to be a risk for schizophrenia in a recent meta-analysis (Mostaid et al., 2017), which might be due to the inclusion of a family study (Yang et al., 2003) and a case-control study (Naz et al., 2011) that we had excluded. Contrary to Mostaid et al (Mostaid et al., 2017), we excluded Naz et al. (Naz et al., 2011) from our meta-analysis because of a broad and unreliable 95% CI, but included

another study Ma et al. (Ma et al., 2014). *NRG1* rs10503929, rs2954041 and SNP8NRG241930 (rs62510682) SNPs are situated in the exon 11, intron 5, and 5'-UTR of the *NRG1* gene respectively (Mostaid et al., 2017). Nevertheless, the functional role of these *NRG1* SNPs in the development of schizophrenia is not well understood.

Although we found significant associations of several *DAO*, *DAOA*, and *NRG1* SNPs with schizophrenia, we are unable to comment on the putative effect of these SNPs on DAO, DAOA, and NRG1 expression, which might ultimately explain the glutamate hypothesis of schizophrenia based on the NMDA receptor hypofunction. Future studies should concentrate on associations of *DAO*, *DAOA*, and *NRG1* SNPs with Research Domain Criteria (RDoC) constructs such as positive valence, negative valence, and cognitive systems (Insel et al., 2010; Cuthbert and Insel, 2013) in schizophrenia patients. This approach might help to better understand the role of these SNPs in the development of schizophrenia symptomatology.

Results of this meta-analysis need to be interpreted within its limitations. Although this is the largest and most comprehensive meta-analysis of the association of *DAO*, *DAOA*, and *NRG1* SNPs with schizophrenia to date, the number of studies as well as the sample size for most SNPs were still modest, making results vulnerable to type I or type II error. The overall power of this meta-analysis was not very high, which lead to some false positive/negative results. Thus, the results of this meta-analysis must be interpreted with caution. There were insufficient studies in ethnic groups other than Caucasian and Asian, and the ethnically stratified subgroup analyses were based on even smaller samples. Although we corrected for multiple comparisons, we still focussed on non-corrected p-values, having performed multiple meta-analyses for all available SNPs in the three-targeted genes, rendering results of even significant meta-analyses exploratory. Furthermore, not all studies used research interviews for diagnostic ascertainment, likely introducing imprecision. The results were mostly heterogeneous across studies, which we addressed by using random-effects models (Borenstein et al., 2010). Finally, similar to previous meta-analyses (Tan et al., 2014; Mostaid et al., 2017), we did not include PGC SCZ or other GWAS results in our meta-analysis which included only studies that met the inclusion criteria. This was because of the limitation in the available meta-analytic methods. Currently, to our knowledge there are no meta-analytic methods available to combine GWAS data with usual case-control association studies after weighting the different approaches in a correct manner. Future methodological advances may make such integration of

data possible. Nevertheless, despite these limitations, our systematic review and meta-analysis is the most recent one, having included studies published until March 21, 2017 that assessed the association of *DAO*, *DAOA*, and *NRG1* SNPs with schizophrenia, thus adding 6 (for *DAO* SNPs), 5 (for *DAOA* SNPs), and 18 (for *NRG1* SNPs) more studies to those previously included in the SZGene meta-analysis (Allen et al., 2008).

In summary, this updated meta-analysis focussing on *DAO*, *DAOA*, and *NRG1* SNPs implicated in the glutamate hypothesis of schizophrenia identified 1 schizophrenia risk allele [*DAOA* (rs778293, in Asian samples)] as well as 4 protective alleles [*DAO* (rs4623951, across all studies), *DAOA* (rs3916971, across all studies) and *NRG1* (rs10503929, across all studies and in Caucasian samples, and SNP8NRG241930 (rs62510682), across all studies and in Caucasian samples). Nevertheless, these findings should still be considered with caution since sample sizes were mostly modest and most results showed significant heterogeneity between studies. Therefore, additional, well conducted and large case-control studies of *DAO*, *DAOA*, and *NRG1* SNPs in schizophrenia patients are needed, ideally, linking genetic data to clinical and endophenotypic and biological information related to disease mechanisms and putative gene effects, before the role of *DAO*, *DAOA*, and *NRG1* SNPs in the development and phenotypic expression of schizophrenia can be clarified further.

5.2.6 Supplementary Information

Supplementary Table S1: Excluded studies in the *DAO* meta-analyses and reasons for exclusion

Serial No.	References	Reasons
1	(Fallin et al., 2005)	Family study
2	(Liu et al., 2006)	Family study
3	(Goldberg et al., 2006)	Family study
4	(Chung et al., 2007)	No healthy controls
5	(Stefanis et al., 2007)	Allele and/or genotype frequencies and/or odds ratio not available
6	(Corvin et al., 2007a)	Allele and/or genotype frequencies and/or odds ratio not available
7	(Wirgenes et al., 2009)	No healthy controls
8	(Roussos et al., 2011)	No schizophrenia cases
9	(Yang et al., 2013)	Same population as Liu (Liu et al., 2004)
10	(Sprooten et al., 2015)	*DAO* SNPs not studied
11	(Luykx et al., 2015)	*DAO* SNPs not studied

Supplementary Table S2: Characteristics of included studies in the *DAO* meta-analyses

Serial No.	References	Cases/Controls (N)	Ancestry (Caucasian/Asian/Others)
1	(Chumakov et al., 2002)	213/241	Caucasian
2	(Liu et al., 2004)	547/536	Asian
3	(Schumacher et al., 2004)	299/300	Caucasian
4	(Yamada et al., 2005)	50/52	Asian
		570/570	Asian
5	(Shinkai et al., 2007)	168/168	Caucasian
6	(Wood et al., 2007)	311/291	Caucasian
7	(Corvin et al., 2007b)	373/812	Caucasian
8	(Vilella et al., 2008)	589/615	Caucasian
9	(Jönsson et al., 2009)	837/1473	Caucasian
10	(Ohnuma et al., 2009)	340/340	Asian
11	(Bass et al., 2009)	431/443	Caucasian
12	(Suliman et al., 2010)	531/755	Caucasian
13	(Ohnuma et al., 2010)	1656/1842	Asian
14	(Kim et al., 2010)	448/337	Asian
15	(Papagni et al., 2011)	40/48	Caucasian
16	(Mechelli et al., 2012)	40/47	Caucasian
17	(Sacchetti et al., 2013)	391/488	Caucasian
18	(Liu et al., 2016)	912/600	Asian
19	(Kartalci and Acar, 2016)	96/100	Others (Turkish)
20	(Chu et al., 2017)	248/267	Asian

Supplementary Table S3: Tests for publication bias in the *DAO* meta-analyses

DAO SNP ID	Minor allele	Number of studies	Tests for publication bias					
			Begg's test		Egger's regression test			
			z-value	p-value	Standard error	95% CI	t-value	p-value
rs4623951	C	4	1.02	0.31	0.53	-1.28-3.27	1.87	0.20
rs2111902	G	14	0.88	0.38	1.25	-1.36-4.10	1.09	0.30
rs3918346	T	12	0.69	0.49	1.64	-2.35-4.94	0.79	0.45
rs3741775	G	14	0.33	0.74	1.49	-2.67-3.81	0.38	0.71
rs3918347	G	5	1.71	0.09	1.10	-0.85-6.17	2.41	0.09
rs3825251	G	7	0.3	0.76	1.17	-2.35-3.66	0.56	0.60
rs2070586	A	6	1.13	0.26	3.49	-7.25-12.14	0.70	0.52
rs2070587	G	6	0	1.00	3.64	-9.32-10.90	0.22	0.84

*$p<0.05$ (**bold font**)

Supplementary Table S4: Excluded studies in the *DAOA* meta-analyses and reasons for exclusion

Serial No.	References	Reasons
1	(Addington et al., 2007)	No healthy controls
2	(Hall et al., 2004)	Family study
3	(Fallin et al., 2005)	Family study
4	(Mulle et al., 2005)	Family study
5	(Zou et al., 2005)	Family study
6	(Goldberg et al., 2006)	Family study
7	(Hong et al., 2006)	Family study
8	(Korostishevsky et al., 2006)	Family study
9	(Liu et al., 2006)	Family study
10	(Mattai et al., 2006)	Family study
11	(Yue et al., 2006)	Same population as Yue (Yue et al., 2007)
12	(Chung et al., 2007)	No healthy controls
13	(Donohoe et al., 2007)	95% confidence interval for the odds ratio not available
14	(Nicodemus et al., 2007)	Allele and/or genotype frequencies and/or odds ratio not available
15	(Stefanis et al., 2007)	No schizophrenia cases
16	(Hall et al., 2008)	No schizophrenia cases
17	(Opgen-Rhein et al., 2008)	Allele and/or genotype frequencies and/or odds ratio not available
18	(Soronen et al., 2008)	Family study
19	(Kotaka et al., 2009)	No schizophrenia cases
20	(Ma et al., 2009)	Family study
21	(Maziade et al., 2009)	*DAOA* SNPs not studied
22	(Shi et al., 2009)	Allele and/or genotype frequencies and/or odds ratio not available
23	(Carrera et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
24	(Hartz et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
25	(Huang et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
26	(Jansen et al., 2010)	No schizophrenia cases
27	(Mössner et al., 2010)	No schizophrenia cases
28	(Pae et al., 2010)	No healthy controls
29	(Li et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
30	(Chiesa et al., 2011)	Allele and/or genotype frequencies and/or odds ratio not available
31	(Müller et al., 2011)	Family study
32	(Nixon et al., 2011)	No schizophrenia cases
33	(Schultz et al., 2011)	Allele and/or genotype frequencies and/or odds ratio not available
34	(Graw et al., 2012)	*DAOA* SNPs not studied
35	(Prata et al., 2012)	No schizophrenia cases
36	(Bousman et al., 2013)	No schizophrenia cases
37	(Chiesa et al., 2013)	Allele and/or genotype frequencies and/or odds ratio not available
38	(Pauli et al., 2013)	No schizophrenia cases
39	(Andreou et al., 2015)	No schizophrenia cases
40	(Nickl-Jockschat et al., 2015)	No schizophrenia cases
41	(Soler et al., 2016)	Family study

Supplementary Table S5: Characteristics of included studies in the *DAOA* meta-analyses

Serial No.	References	Cases/Controls (N)	Ancestry (Caucasian/Asian/Others)
1	(Chumakov et al., 2002)	213/241	Caucasian
		183/183	Caucasian
2	(Korostishevsky et al., 2006)	60/130	Caucasian
3	(Schumacher et al., 2004)	299/300	Caucasian
4	(Wang et al., 2004)	537/538	Asian
5	(Ma et al., 2006)	588/588	Asian
		183/182	Caucasian
6	(Williams et al., 2006)	709/1416	Caucasian
7	(Bakker et al., 2007)	308/467	Caucasian
8	(Corvin et al., 2007b)	373/812	Caucasian
9	(Shin et al., 2007)	388/367	Asian
10	(Shinkai et al., 2007)	168/168	Caucasian
11	(Wood et al., 2007)	311/291	Caucasian
12	(Yue et al., 2007)	359/359	Asian
13	(Sanders et al. 2008)	1678/2002	Caucasian
14	(Vilella et al., 2008)	589/615	Caucasian
15	(Bass et al., 2009)	431/443	Caucasian
16	(Jönsson et al., 2009)	837/1473	Caucasian
17	(Ohi et al., 2009)	1774/2092	Asian
18	(Réthelyi et al., 2010)	284/238	Caucasian
19	(Mechelli et al., 2012)	40/47	Caucasian
20	(Chen et al., 2013)	454/480	Asian
21	(Sacchetti et al., 2013)	391/488	Caucasian
22	(Kartalci and Acar, 2016)	96/100	Others (Turkish)
23	(Chu et al., 2017)	248/267	Asian

Supplementary Table S6: Tests for publication bias in the *DAOA* meta-analyses

DAOA SNP ID	Minor allele	Number of studies	Tests for publication bias					
			Begg's test		Egger's regression test			
			z-value	p-value	Standard error	95% CI	t-value	p-value
rs3916965 (M12)	A	16	-0.14	0.89	0.96	-1.9-2.2	0.16	0.88
rs3916966 (M13)	C	6	0	1.00	0.79	-2.19-2.17	-0.02	0.98
rs3916967 (M14)	G	14	0	1.00	0.74	-1.22-2.02	0.54	0.60
rs2391191 (M15)	A	22	-0.56	0.57	0.90	-2.39-1.34	-0.59	0.56
rs947267 (M18)	C	11	0.31	0.76	1.81	-1.85-6.35	1.24	0.25
rs778294 (M19)	T	14	-0.44	0.66	0.7	-1.8-1.27	-0.38	0.71
rs3916970 (M20)	A	5	-0.73	0.46	0.33	-1.51-0.57	-1.45	0.24
rs3916971 (M21)	T	4	-0.34	0.73	1.56	-6.61-6.83	0.07	0.95
rs778293 (M22)	G	13	-0.79	0.43	1.17	-3.47-1.69	-0.76	0.46
rs3918342 (M23)	T	19	0.21	0.83	1.05	-2.49-1.93	-0.27	0.79
rs1421292 (M24)	A	5	-0.24	0.81	1.31	-4.28-4.03	-0.095	0.93
rs9558562	G	3	0	1.00	1.20	-15.96-14.6	-0.57	0.67

*$p<0.05$ (**bold font**)

Supplementary Table S7: Excluded studies in the *NRG1* meta-analyses and reasons for exclusion

Serial No.	References	Reasons
1	(Yang et al., 2003)	Family study
2	(Hall et al., 2004)	Family study
3	(Tang et al., 2004)	Allele and/or genotype frequencies and/or odds ratio not available
4	(Thiselton et al., 2004)	Family study
5	(Costas et al., 2005)	No schizophrenia cases
6	(Duan et al., 2005)	Family study
7	(Lin et al., 2005)	No schizophrenia cases
8	(Liu et al., 2005)	Family study
9	(Gardner et al., 2006)	No schizophrenia cases
10	(Hall et al., 2006)	No schizophrenia cases
11	(Law et al., 2006)	Allele and/or genotype frequencies and/or odds ratio not available
12	(Norton et al., 2006)	Allele and/or genotype frequencies and/or odds ratio not available
13	(Walss-Bass et al., 2006b)	Allele and/or genotype frequencies and/or odds ratio not available
14	(Walss-Bass et al., 2006a)	Family study
15	(Addington et al., 2007)	No schizophrenia cases
16	(Mathew et al., 2007)	Allele and/or genotype frequencies and/or odds ratio not available
17	(Rosa et al., 2007)	Family study
18	(Thomson et al., 2007)	Allele and/or genotype frequencies and/or odds ratio not available
19	(Turunen et al., 2007)	Family study
20	(Georgieva et al., 2008)	Family study
21	(Gruber et al., 2008)	Family study
22	(Mechelli et al., 2008)	Allele and/or genotype frequencies and/or odds ratio not available
23	(Winterer et al., 2008)	No schizophrenia cases
24	(Dutt et al., 2009)	Allele and/or genotype frequencies and/or odds ratio not available
25	(Keri et al., 2009)	No schizophrenia cases
26	(Kéri et al., 2009)	Family study
27	(Kircher et al., 2009)	Allele and/or genotype frequencies and/or odds ratio not available
28	(Mata et al., 2009)	Allele and/or genotype frequencies and/or odds ratio not available
29	(Okochi et al., 2009)	No schizophrenia cases
30	(van Schijndel et al., 2009)	Allele and/or genotype frequencies and/or odds ratio not available
31	(Mata et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
32	(Parlapani et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
33	(Shibuya et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
34	(Walker et al., 2010)	Allele and/or genotype frequencies and/or odds ratio not available
35	(Garcia-Barceló et al., 2011)	Allele and/or genotype frequencies and/or odds ratio not available
36	(Greenwood et al., 2011)	Family study
37	(Naz et al., 2011)	95% confidence interval for the odds ratio is very broad
38	(Cannon et al., 2012)	No schizophrenia cases
39	(Greenwood et al., 2012)	95% confidence interval for the odds ratio not given
40	(Tosato et al., 2012)	Allele and/or genotype frequencies and/or odds ratio not available
41	(Walshe et al., 2012)	No schizophrenia cases
42	(Yokley et al., 2012)	Family study
43	(Bousman et al., 2013)	No schizophrenia cases
44	(Loh et al., 2013)	Allele and/or genotype frequencies and/or odds ratio not available
45	(Yang et al., 2013)	Allele and/or genotype frequencies and/or odds ratio not available
46	(Grimm et al., 2014)	Family study
47	(Nawaz et al., 2014)	Allele and/or genotype frequencies and/or odds ratio not available
48	(Tosato et al., 2014)	No healthy controls
49	(Cho et al., 2015)	Allele and/or genotype frequencies and/or odds ratio not available
50	(Suárez-Pinilla et al., 2015)	No schizophrenia cases
51	(Greenwood et al., 2016)	Family study
52	(Jajodia et al., 2016)	Same population as Jajodia (Jajodia et al., 2015)

Supplementary Table S8: Characteristics of included studies in the *NRG1* meta-analyses

Serial No.	References	Case/Control (N)	Ancestry (Caucasian/Asian/Others)
1	(Stefansson et al., 2002)	478/394	Caucasian
2	(Stefansson et al., 2003)	609/618	Caucasian
3	(Williams et al., 2003)	573/618	Caucasian
4	(Bakker et al., 2004)	260/585	Caucasian
5	(Corvin et al., 2004)	243/222	Caucasian
6	(Hong et al., 2004)	228/269	Asian
7	(Iwata et al., 2004)	607/515	Asian
8	(Kampman et al., 2004)	94/395	Caucasian
9	(Li et al., 2004)	298/336	Asian
10	(Zhao et al., 2004)	369/299	Asian
11	(Petryshen et al., 2005)	321/242	Caucasian
12	(Fukui et al., 2006)	349/424	Asian
13	(Ingason et al., 2006)	325/353	Caucasian
14	(Kim et al., 2006)	242/242	Asian
15	(Lachman et al., 2006)	177/164	Caucasian
		141/142	Asian
16	(Benzel et al., 2007)	396/1342	Caucasian
17	(Hänninen et al., 2007)	113/393	Caucasian
18	(Bramon et al., 2008)	60/34	Caucasian
19	(Crowley et al., 2008)	738/733	Others (Mixed)
20	(Hong et al., 2008)	244/186	Asian
21	(Ikeda et al., 2008)	1126/1022	Asian
22	(Sanders et al., 2008)	1870/2002	Caucasian
23	(Shiota et al., 2008)	416/520	Asian
24	(Vilella et al., 2008)	589/617	Caucasian
25	(Alaerts et al., 2009)	486/514	Caucasian
26	(Jönsson et al., 2009)	837/1473	Caucasian
27	(Nicodemus et al., 2009)	296/365	Others (Mixed)
28	(Wang et al., 2009)	31/36	Asian
29	(Réthelyi et al., 2010)	280/230	Caucasian
30	(Squassina et al., 2010)	171/349	Caucasian
31	(Shariati et al., 2011)	95/95	Caucasian
32	(Moon et al., 2011)	273/479	Others (Costa Rican)
33	(Papiol et al., 2011)	1071/1056	Caucasian
34	(Crisafulli et al., 2012)	221/170	Asian
35	(Kang et al., 2012)	287/120	Asian
36	(Kim et al., 2012)	435/390	Asian
37	(Yang, 2012)	221/359	Asian
38	(Weickert et al., 2012)	37/37	Caucasian
39	(Kukshal et al., 2013)	1007/1019	Asian
40	(Díez et al., 2014)	31/23	Caucasian
41	(Ma et al., 2014)	976/1043	Asian
42	(Thirunavukkarasu et al., 2014)	38/37	Asian
43	(Gutiérrez-Fernández et al., 2014)	215/650	Caucasian
44	(Jajodia et al., 2015)	436/401	Asian
		351/385	Asian
45	(Terzić et al., 2015)	138/94	Caucasian
46	(He et al., 2016)	248/236	Asian
47	(Wen et al., 2016)	1248/1248	Asian
48	(Yoshimi et al., 2016)	59/60	Asian

Supplementary Table S9: Tests for publication bias in the *NRG1* meta-analyses

***NRG1* SNP ID**	**Minor allele**	**Number of studies**	**Tests for publication bias**					
			Begg's test		**Egger's regression test**			
			z-value	**p-value**	**Standard error**	**95% CI**	**t-value**	**p-value**
SNP8NRG241930 (rs62510682)	T	22	-0.28	0.78	0.57	-1.52-0.85	-0.58	0.57
rs10503929	C	4	-0.34	0.73	0.11	-0.49-0.44	-0.23	0.84
rs6994992	T	25	0.07	0.94	0.73	-1.55-1.48	-0.05	0.96
rs35753505	C	29	-0.69	0.49	0.56	-1.67-0.62	-0.94	0.36
rs6988339	G	6	0.75	0.45	1.93	-4.75-5.96	0.31	0.77
SNP8NRG221132 (rs73235619)	A	8	-0.62	0.54	0.84	-3.32-0.79	-1.51	0.18
rs3924999	A	13	-1.53	0.13	0.61	-2.11-0.58	-1.25	0.24
SNP8NRG433E1006 (rs113317778)	A	6	1.13	0.26	1.36	-3.2-4.33	0.42	0.70
rs2954041	T	6	0	1.00	0.87	-2.05-2.8	0.43	0.69
rs776401	C	4	-0.34	0.73	3.08	-15.3-11.19	-0.67	0.57
rs2439272	A	5	-0.73	0.46	2.35	-9.87-5.07	-1.02	0.38
rs2466058	T	4	0.34	0.73	3.11	-12.2-14.59	0.38	0.74
rs4733376	G	5	0.24	0.81	2.16	-4.72-9.06	1	0.39
rs7014762	A	3	1.04	0.30	2.30	-26.9-31.44	0.99	0.50

*$p<0.05$ (**bold font**)

Supplementary Table S10: Comparison of our meta-analysis of *DAO*, *DAOA*, and *NRG1* SNPs with Psychiatric Genomics Consortium (PGC) 2014 genome-wide association study (GWAS) data

Gene	SNP ID	PGC GWAS				Our meta-analysis		
		Reference allele for OR[a]	OR	SE	p-value	Minor allele	OR	p-value
D-amino acid oxidase (*DAO*)	rs4623951	T	0.98708	0.0116	0.2628	C	0.88	**0.02***
	rs2111902	T	0.96696	0.0123	**0.006262***	G	1.02	0.74
	rs3918346	T	1.02922	0.013	**0.02661***	T	1.00	0.97
	rs3741775	T	1.02665	0.0114	**0.02091***	G	1.01	0.93
	rs3918347	A	0.97609	0.0123	**0.04916***	G	1.11	0.18
	rs3825251	A	0.98728	0.0151	0.3961	G	1.02	0.5
	rs2070586	A	1.0321	0.0149	**0.03354***	A	1.04	0.65
	rs2070587	T	0.96841	0.0141	**0.02247***	G	1.00	0.99
D-amino acid oxidase activator (*DAOA*)	rs3916965 (M12)	A	0.9997	0.0113	0.9801	A	0.98	0.67
	rs3916966 (M13)	A	1.0024	0.0112	0.8301	C	0.95	0.17
	rs3916967 (M14)	A	1.0011	0.0112	0.9191	G	0.999	0.96
	rs2391191 (M15)	A	0.9992	0.0112	0.9452	A	1.02	0.65
	rs947267 (M18)	A	0.99154	0.011	0.4398	C	1.04	0.51
	rs778294 (M19)	T	1.0008	0.0119	0.945	T	0.97	0.3
	rs3916970 (M20)	A	1.00552	0.0117	0.6362	A	1.06	0.14
	rs3916971 (M21)	T	0.99551	0.011	0.6779	T	0.84	**0.01***
	rs778293 (M22)	A	1.00582	0.0111	0.5972	G	1.04	0.35
	rs3918342 (M23)	T	1.0007	0.0108	0.9464	T	1.00	0.95
	rs1421292 (M24)	A	1.0024	0.0109	0.8248	A	0.95	0.30
	rs9558562	A	2.42202	1.047	0.3981	G	0.93	0.47
Neuregulin1 (*NRG1*)	SNP8NRG241930 (rs62510682)	T	0.97424	0.0118	**0.02719***	T	0.95	**0.04***
	rs10503929	T	1.01959	0.014	0.1641	C	0.89	**0.01***
	rs6994992	T	1.01005	0.0115	0.385	T	1.02	0.56
	rs35753505	T	0.99293	0.0113	0.5285	C	1.04	0.14
	rs6988339	A	1.0027	0.0111	0.8079	G	0.995	0.93
	SNP8NRG221132 (rs73235619)	A	0.98748	0.0185	0.4959	A	0.98	0.62
	rs3924999	A	1.01542	0.0112	0.1726	A	0.98	0.34
	SNP8NRG433E1006 (rs113317778)	A	1.0023	0.0212	0.912	A	0.98	0.87
	rs2954041	T	1.01329	0.0449	0.7694	T	1.05	0.34
	rs776401	T	1.0031	0.0112	0.7852	C	0.97	0.69
	rs2439272	A	1.002	0.0114	0.8635	A	0.82	0.06
	rs2466058	T	1.04175	0.0205	**0.0458***	T	1.08	0.47
	rs4733376	A	0.96069	0.0175	**0.02182***	G	1.04	0.57
	rs7014762	A	1.01867	0.0129	0.1523	A	1.04	0.47

*p<0.05 (**bold font**); #p<0.0015 (*italics*) (significant with Bonferroni correction); [a] Reference allele is not necessarily a minor allele, OR: Odds ratio; SE: standard error of ln(OR))

Supplementary Table S11: Power analysis for *DAO* meta-analyses

DAO SNP ID	Minor allele	All studies							Caucasian studies							Asian studies						
		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]	
		Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)	Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)	Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)
rs4623951	C	1554	1589	0.37	0.40	0.53	3259	3259	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2111902	G	4394	5204	0.42	0.41	0.13	119950	119950	2791	3606	0.34	0.34	0.10	173420	173420	1507	1498	0.70	0.69	0.12	40879	40879
rs3918346	T	3907	4617	0.33	0.33	0.06	$1.1x10^7$	$1.1x10^7$	2400	3119	0.28	0.27	0.13	68480	68480	1507	1498	0.52	0.52	0.07	342952	342952
rs3741775	G	5066	6085	0.45	0.45	0.08	764460	764460	3215	4220	0.46	0.46	0.07	767623	767623	1755	1765	0.28	0.28	0.07	619093	619093
rs3918347	G	1560	1574	0.45	0.42	0.43	4500	4500	772	897	0.40	0.35	0.57	1569	1569	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3825251	G	3220	4078	0.31	0.30	0.1	163818	163818	1713	2580	0.14	0.13	0.16	29191	29191	1507	1498	0.49	0.49	0.09	123585	123585
rs2070586	A	2967	2872	0.26	0.26	0.17	37172	37172	1267	1595	0.15	0.14	0.19	15591	15591	1700	1277	0.40	0.40	0.10	60588	60588
rs2070587	G	3724	4049	0.24	0.24	0.05	$2.3x10^8$	$2.3x10^8$	1817	2576	0.19	0.19	0.11	76578	76578	1817	2576	0.18	0.19	0.11	75040	75040

[a] A-priori z-test for proportions; P1: Proportion of cases carrying minor allele; P2: Proportion of controls carrying minor allele

Supplementary Table S12: Power analysis for *DAOA* meta-analyses

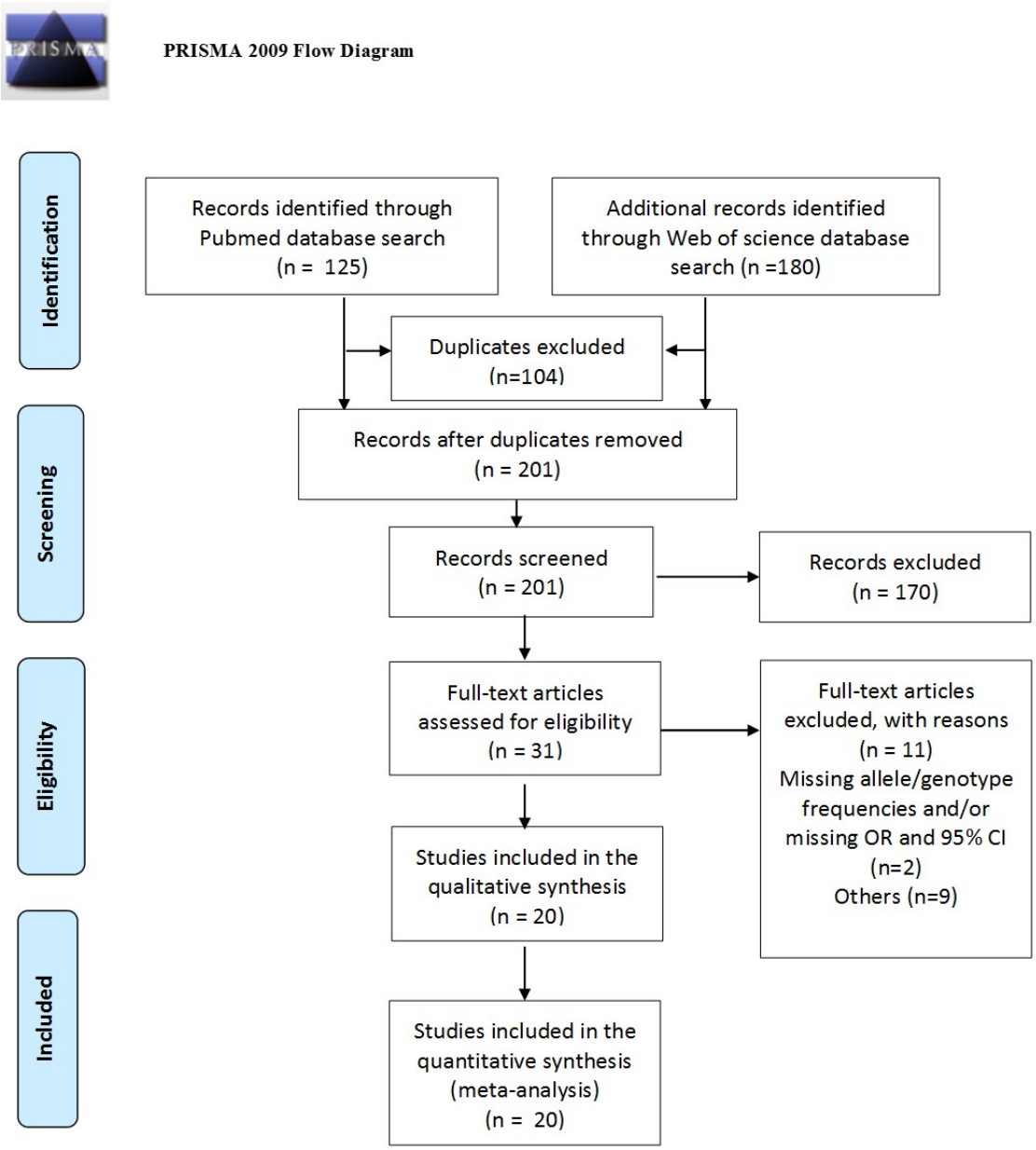
DAOA SNP ID	Minor allele	All studies							Caucasian studies							Asian studies						
		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]	
		Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)	Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)	Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)
rs3916965 (M12)	A	8286	10208	0.49	0.50	0.17	123641	123641	4297	5876	0.36	0.37	0.34	19948	19948	3989	4332	0.62	0.61	0.16	59836	59836
rs3916966 (M13)	C	2976	3403	0.45	0.46	0.27	18146	18146	2134	2556	0.41	0.42	0.21	20832	20832	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3916967 (M14)	G	7554	8495	0.54	0.54	0.06	3066792	3066792	3813	4430	0.33	0.33	0.06	2723339	2723339	3741	4065	0.65	0.65	0.07	704190	704190
rs2391191 (M15)	A	10887	13568	0.49	0.48	0.19	123499	123499	6443	8777	0.36	0.37	0.10	318250	318250	4348	4691	0.64	0.63	0.40	14570	14570
rs947267 (M18)	C	3404	3543	0.45	0.44	0.19	37676	37676	1955	2070	0.49	0.49	0.09	123615	123615	1449	1473	0.37	0.37	0.10	58920	58920
rs778294 (M19)	T	8364	10558	0.22	0.22	0.21	85012	85012	4801	6672	0.29	0.29	0.12	158142	158142	3563	3886	0.14	0.14	0.15	59728	59728
rs3916970 (M20)	A	2618	3013	0.6	0.58	0.28	15237	15237	456	554	0.40	0.39	0.09	36622	36622	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3916971 (M21)	T	844	921	0.44	0.48	0.57	1659	1659	456	554	0.40	0.43	0.32	2190	2190	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs778293 (M22)	G	7486	9409	0.36	0.35	0.33	35020	35020	456	554	0.41	0.41	0.06	332222	332222	2899	3218	0.31	0.28	0.88	2349	2349
rs3918342 (M23)	T	9288	11253	0.51	0.51	0.06	6307096	6307096	4587	6191	0.48	0.47	0.06	$1.2x10^7$	$1.2x10^7$	3960	4324	0.54	0.53	0.32	18209	18209
rs1421292 (M24)	A	2138	2830	0.44	0.45	0.23	18074	18074	5328	6929	0.46	0.47	0.50	13673	13673	N/A	N/A	N/A	N/A	N/A	N/A	59836
rs9558562	G	1093	1233	0.27	0.29	0.19	12662	12662	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[a] A-priori z-test for proportions; P1: Proportion of cases carrying minor allele; P2: Proportion of controls carrying minor allele

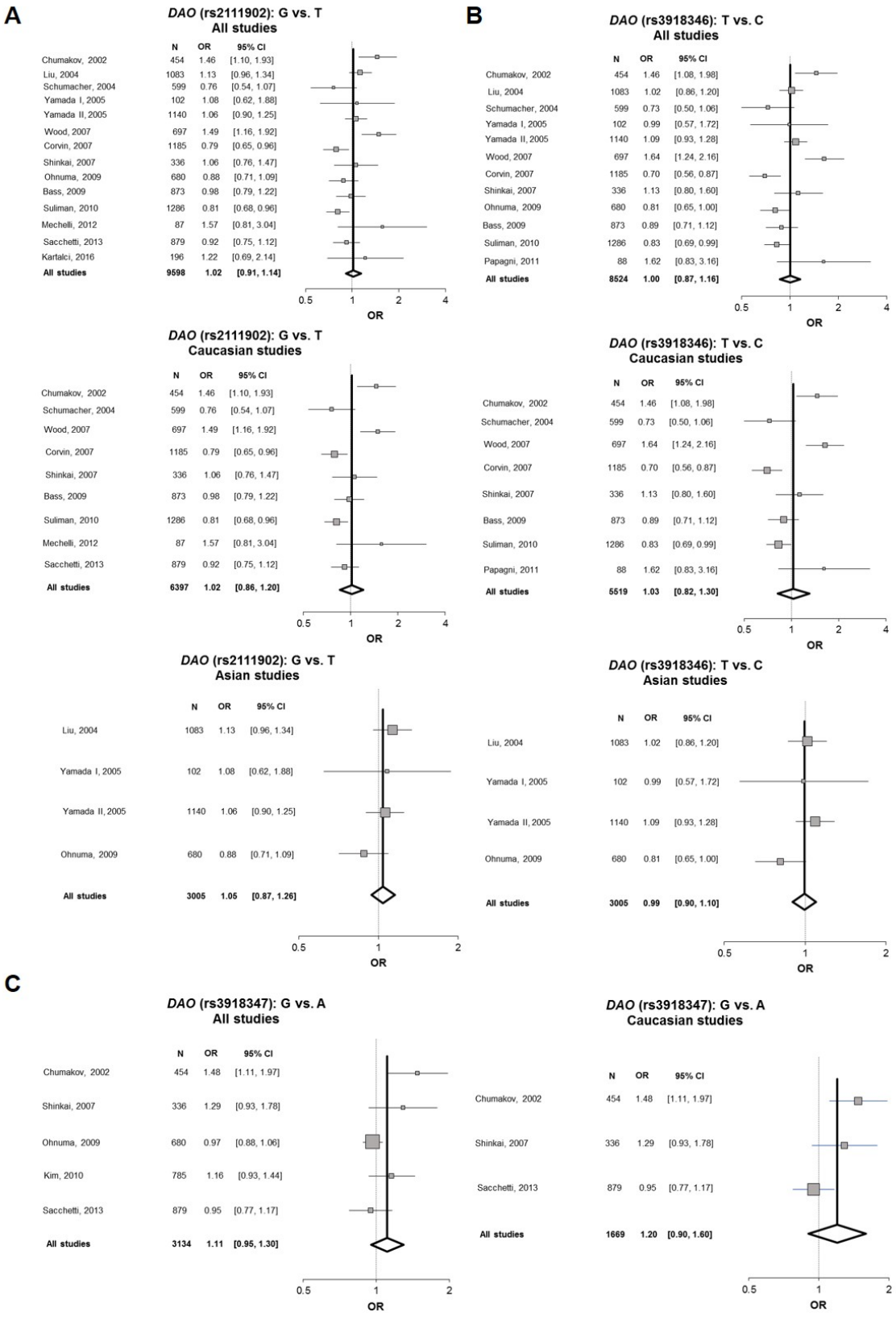
Supplementary Table S13: Power analysis for *NRG1* meta-analyses

NRG1 SNP ID	Minor allele	All studies							Caucasian studies							Asian studies						
		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]		Post-hoc z-test for proportions					Sample size needed to reach 80% power[a]	
		Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)	Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)	Cases (N)	Controls (N)	P1	P2	Power	Cases (N)	Controls (N)
rs62510682	T	10850	12048	0.29	0.30	0.57	21190	21190	7384	8630	0.33	0.35	0.43	22947	22947	3466	3418	0.22	0.22	0.07	523489	523489
rs10503929	C	3089	3755	0.19	0.20	0.59	5990	5990	2845	3569	0.20	0.22	0.59	5612	5612	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs6994992	T	13015	14805	0.42	0.42	0.21	120680	120680	6875	8596	0.34	0.33	0.32	34032	34032	5129	4997	0.55	0.54	0.26	30627	30627
rs35753505	C	11485	12440	0.42	0.41	0.41	37099	37099	7694	8812	0.35	0.33	0.44	22915	22915	3791	3628	0.55	0.54	0.15	62652	62652
rs6988339	G	4227	5385	0.45	0.45	0.07	765091	765091	2587	3586	0.41	0.42	0.20	29998	29998	1640	1799	0.49	0.48	0.17	21447	21447
rs73235619	A	5286	5405	0.09	0.09	0.07	$1.0x10^6$	$1.0x10^6$	5286	5405	0.09	0.09	0.07	$1.0x10^6$	$1.0x10^6$	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs3924999	A	6845	8294	0.54	0.54	0.15	122879	122879	3861	5551	0.44	0.45	0.12	122235	122235	2740	5297	0.76	0.77	0.13	61382	61382
rs113317778	A	4371	4285	0.07	0.07	0.07	831476	831476	3764	3770	0.09	0.09	0.07	$1.0x10^6$	$1.0x10^6$	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2954041	T	4782	6500	0.26	0.26	0.33	23790	23790	3103	4817	0.20	0.20	0.11	122720	122720	1679	1683	0.37	0.35	0.16	23559	23559
rs776401	C	3424	5059	0.47	0.48	0.16	62970	62970	3424	5059	0.47	0.48	0.16	62970	62970	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2439272	A	3279	4299	0.40	0.45	0.99	1314	1314	2587	3586	0.50	0.56	0.99	884	884	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs2466058	T	3733	3786	0.11	0.11	0.25	24803	24803	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs4733376	G	4689	5651	0.27	0.26	0.31	37722	37722	2587	3586	0.11	0.10	0.36	11325	11325	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs7014762	A	1493	1767	0.34	0.33	0.14	34132	34132	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[a] A-priori z-test for proportions; P1: Proportion of cases carrying minor allele; P2: Proportion of controls carrying minor allele



Supplementary Figure S1: PRISMA Flow diagram for literature search of *DAO* SNP data published till March 21, 2017. Search terms were (DAO OR DAAO) AND SCHIZOPHRENIA. OR: odds ratio; CI: confidence interval.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All studies
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Chumakov, 2002 454 1.46 [1.10, 1.93]
Liu, 2004 1083 1.13 [0.96, 1.34]
Schumacher, 2004 599 0.76 [0.54, 1.07]
Yamada I, 2005 102 1.08 [0.62, 1.88]
Yamada II, 2005 1140 1.06 [0.90, 1.25]
Wood, 2007 697 1.49 [1.16, 1.92]
Corvin, 2007 1185 0.79 [0.65, 0.96]
Shinkai, 2007 336 1.06 [0.76, 1.47]
Ohnuma, 2009 680 0.88 [0.71, 1.09]
Bass, 2009 873 0.98 [0.79, 1.22]
Suliman, 2010 1286 0.81 [0.68, 0.96]
Mechelli, 2012 87 1.57 [0.81, 3.04]
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OR
DAO (rs2111902): G vs. T
Caucasian studies
N OR 95% CI
Chumakov, 2002 454 1.46 [1.10, 1.93]
Schumacher, 2004 599 0.76 [0.54, 1.07]
Wood, 2007 697 1.49 [1.16, 1.92]
Corvin, 2007 1185 0.79 [0.65, 0.96]
Shinkai, 2007 336 1.06 [0.76, 1.47]
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OR
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Asian studies
N OR 95% CI
Liu, 2004 1083 1.13 [0.96, 1.34]
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Ohnuma, 2009 680 0.88 [0.71, 1.09]
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All studies
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Schumacher, 2004 599 0.73 [0.50, 1.06]
Yamada I, 2005 102 0.99 [0.57, 1.72]
Yamada II, 2005 1140 1.09 [0.93, 1.28]
Wood, 2007 697 1.64 [1.24, 2.16]
Corvin, 2007 1185 0.70 [0.56, 0.87]
Shinkai, 2007 336 1.13 [0.80, 1.60]
Ohnuma, 2009 680 0.81 [0.65, 1.00]
Bass, 2009 873 0.89 [0.71, 1.12]
Suliman, 2010 1286 0.83 [0.69, 0.99]
Papagni, 2011 88 1.62 [0.83, 3.16]
All studies 8524 1.00 [0.87, 1.16]
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OR
DAO (rs3918346): T vs. C
Caucasian studies
N OR 95% CI
Chumakov, 2002 454 1.46 [1.08, 1.98]
Schumacher, 2004 599 0.73 [0.50, 1.06]
Wood, 2007 697 1.64 [1.24, 2.16]
Corvin, 2007 1185 0.70 [0.56, 0.87]
Shinkai, 2007 336 1.13 [0.80, 1.60]
Bass, 2009 873 0.89 [0.71, 1.12]
Suliman, 2010 1286 0.83 [0.69, 0.99]
Papagni, 2011 88 1.62 [0.83, 3.16]
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OR
DAO (rs3918346): T vs. C
Asian studies
N OR 95% CI
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Yamada I, 2005 102 0.99 [0.57, 1.72]
Yamada II, 2005 1140 1.09 [0.93, 1.28]
Ohnuma, 2009 680 0.81 [0.65, 1.00]
All studies 3005 0.99 [0.90, 1.10]
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OR
C
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All studies
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Shinkai, 2007 336 1.29 [0.93, 1.78]
Ohnuma, 2009 680 0.97 [0.88, 1.06]
Kim, 2010 785 1.16 [0.93, 1.44]
Sacchetti, 2013 879 0.95 [0.77, 1.17]
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OR
DAO (rs3918347): G vs. A
Caucasian studies
N OR 95% CI
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Shinkai, 2007 336 1.29 [0.93, 1.78]
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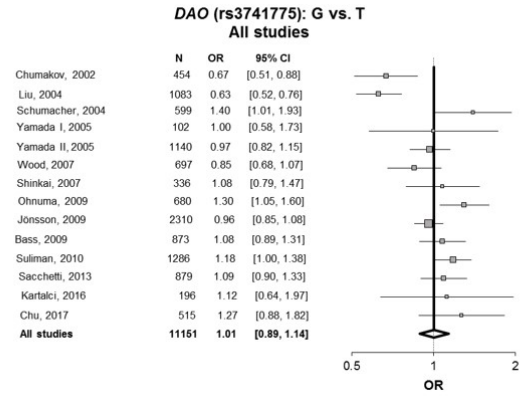
OR

D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Kartalci, 2016 196 1.12 [0.64, 1.97]
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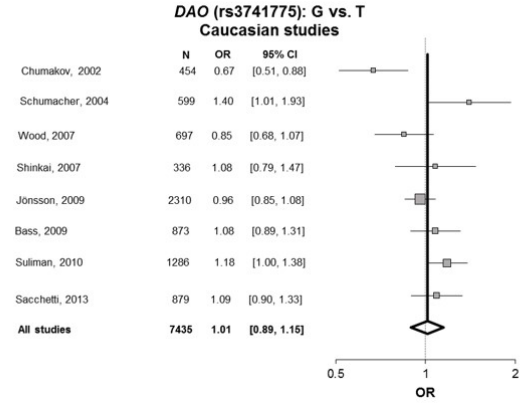
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Wood, 2007 697 0.85 [0.68, 1.07]
Shinkai, 2007 336 1.08 [0.79, 1.47]
Jönsson, 2009 2310 0.96 [0.85, 1.08]
Bass, 2009 873 1.08 [0.89, 1.31]
Suliman, 2010 1286 1.18 [1.00, 1.38]
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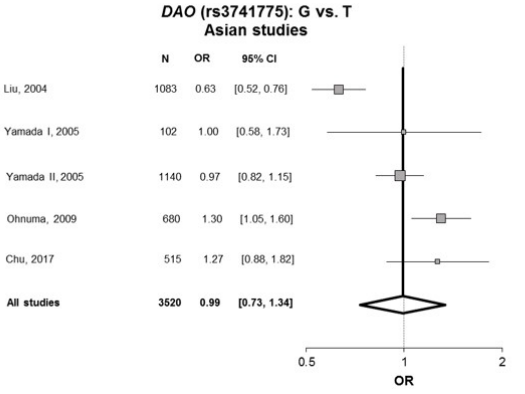
0.5 1 2
OR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Asian studies
N OR 95% CI
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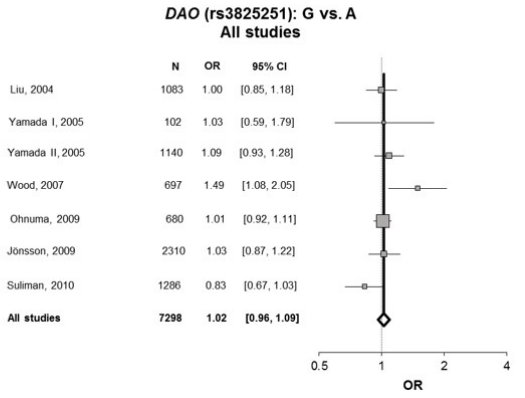
Yamada I, 2005 102 1.00 [0.58, 1.73]
Yamada II, 2005 1140 0.97 [0.82, 1.15]
Ohnuma, 2009 680 1.30 [1.05, 1.60]
Chu, 2017 515 1.27 [0.88, 1.82]
All studies 3520 0.99 [0.73, 1.34]
0.5 1 2
OR

E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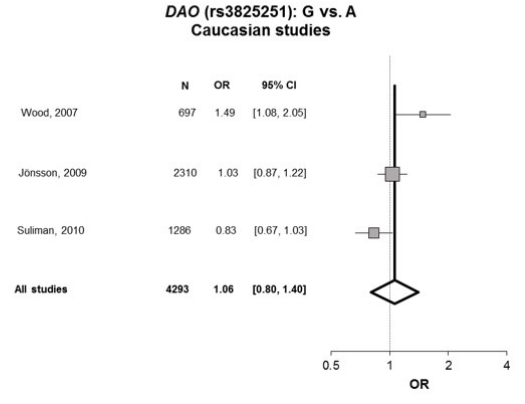
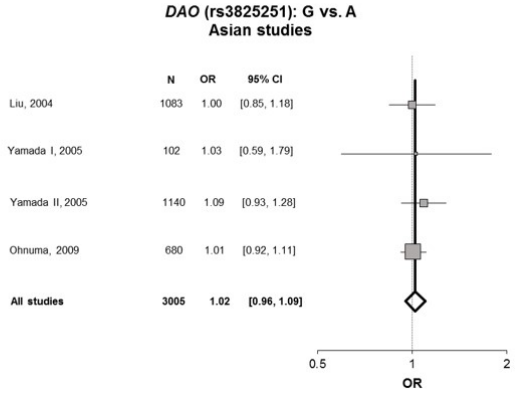
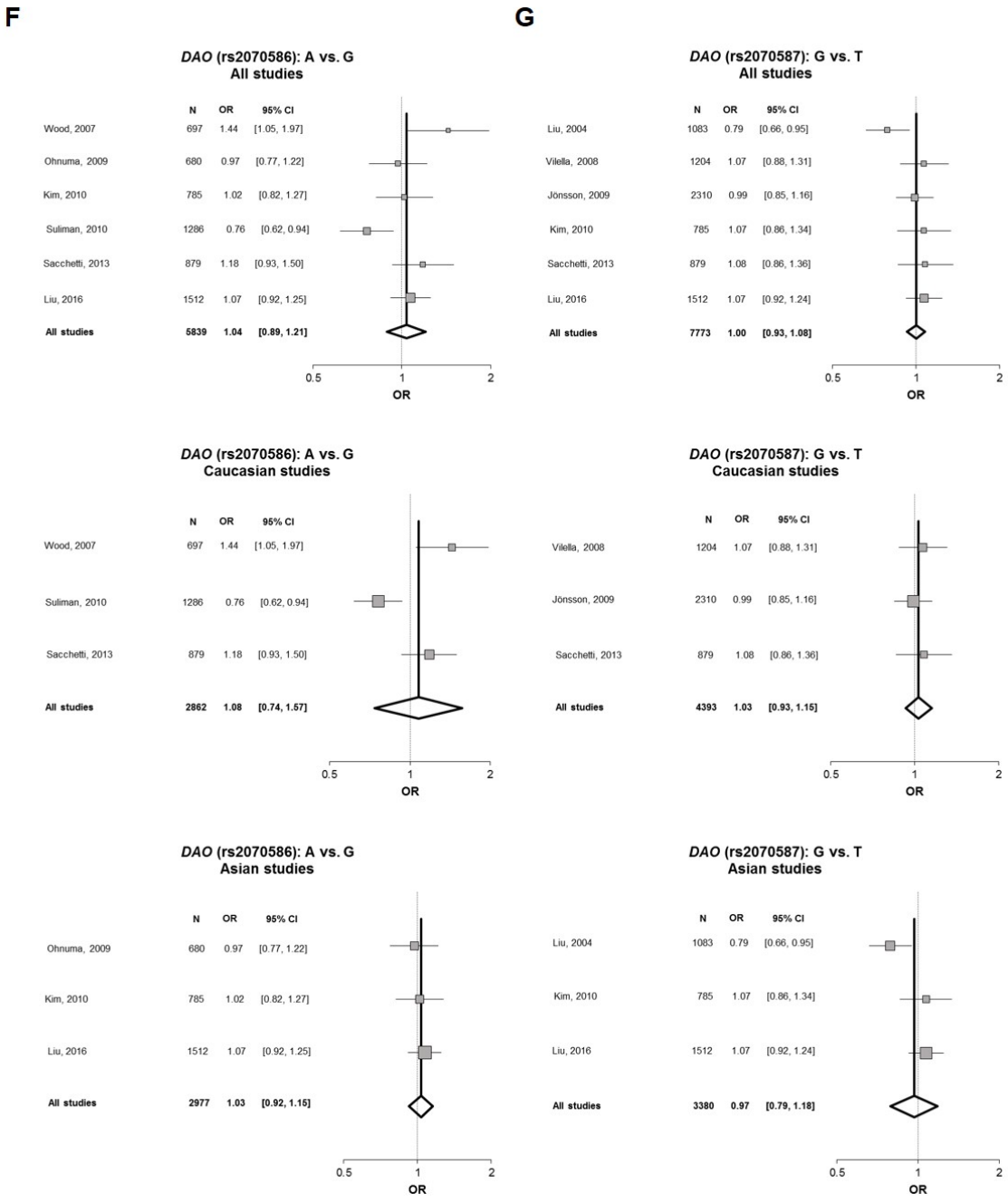
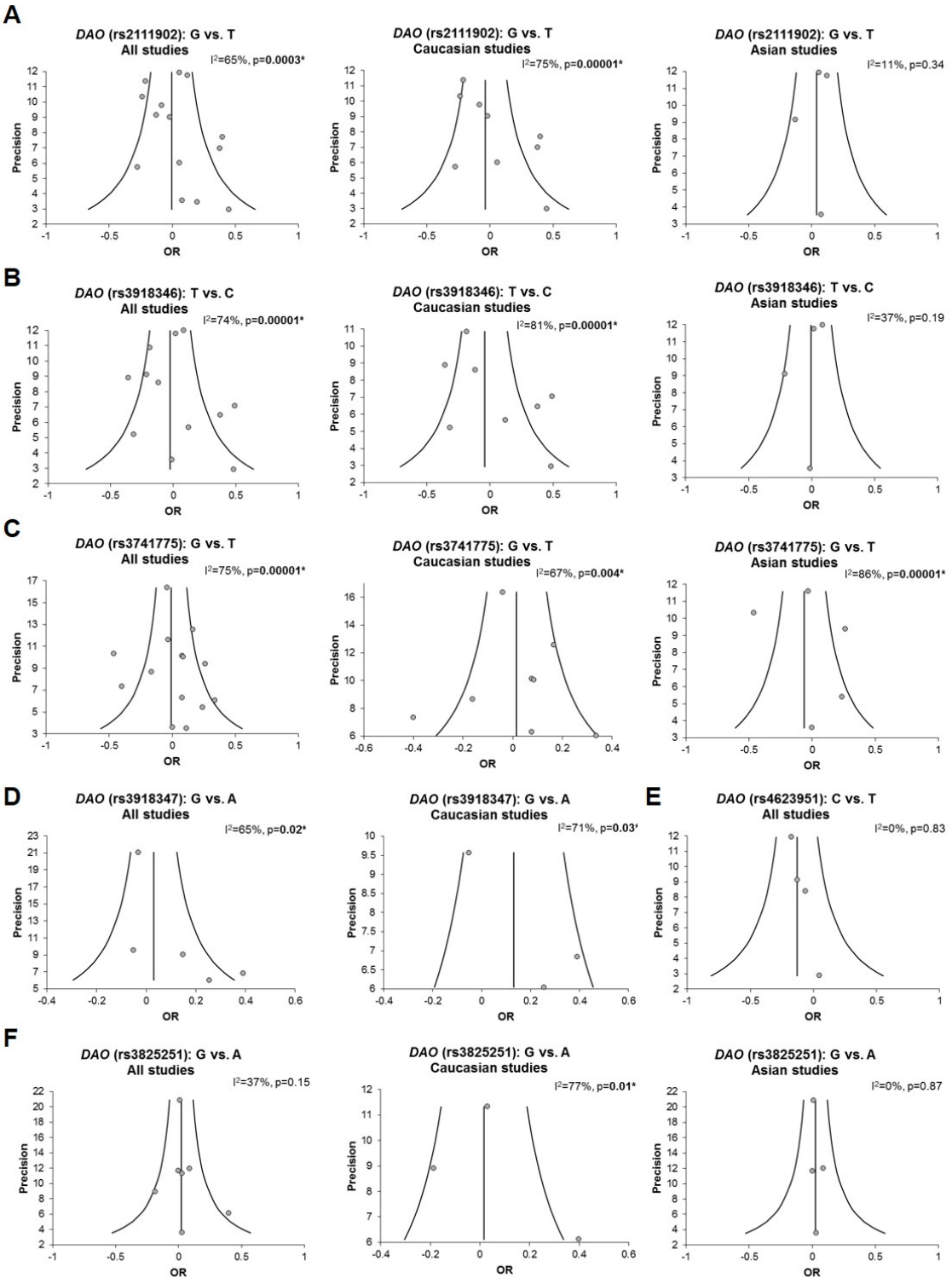
Wood, 2007 697 1.49 [1.08, 2.05]
Ohnuma, 2009 680 1.01 [0.92, 1.11]
Jönsson, 2009 2310 1.03 [0.87, 1.22]
Suliman, 2010 1286 0.83 [0.67, 1.03]
All studies 7298 1.02 [0.96, 1.09]
0.5 1 2 4
OR

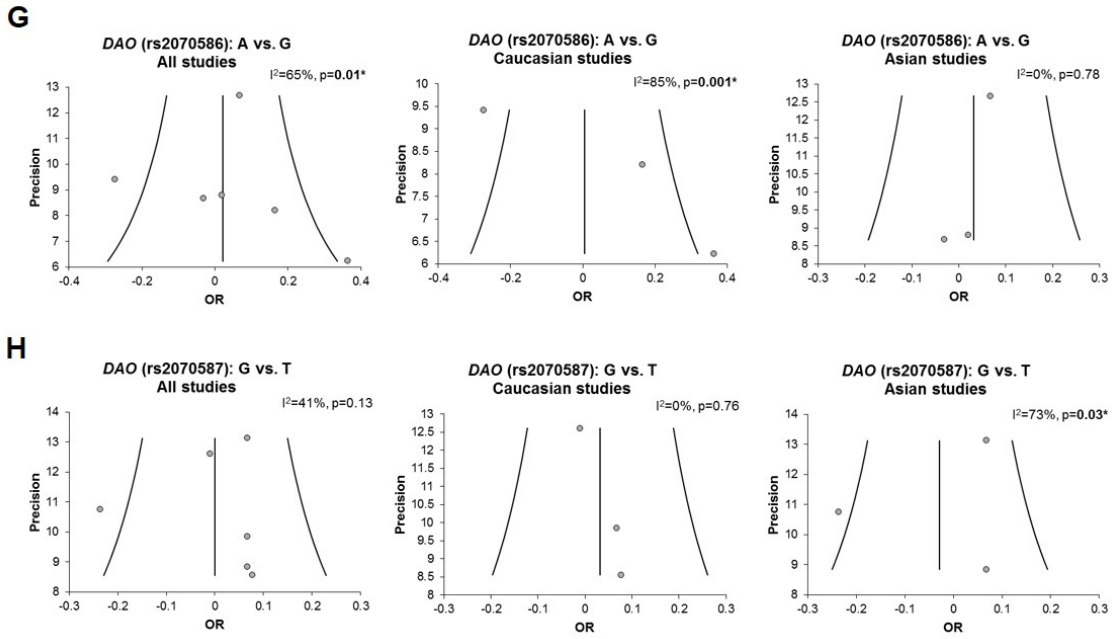

DAO (rs3825251): G vs. A
Caucasian studies
N OR 95% CI
Wood, 2007 697 1.49 [1.08, 2.05]
Jönsson, 2009 2310 1.03 [0.87, 1.22]
Suliman, 2010 1286 0.83 [0.67, 1.03]
All studies 4293 1.06 [0.80, 1.40]
0.5 1 2 4
OR


DAO (rs3825251): G vs. A
Asian studies
N OR 95% CI
Liu, 2004 1083 1.00 [0.85, 1.18]
Yamada I, 2005 102 1.03 [0.59, 1.79]
Yamada II, 2005 1140 1.09 [0.93, 1.28]
Ohnuma, 2009 680 1.01 [0.92, 1.11]
All studies 3005 1.02 [0.96, 1.09]
0.5 1 2
OR

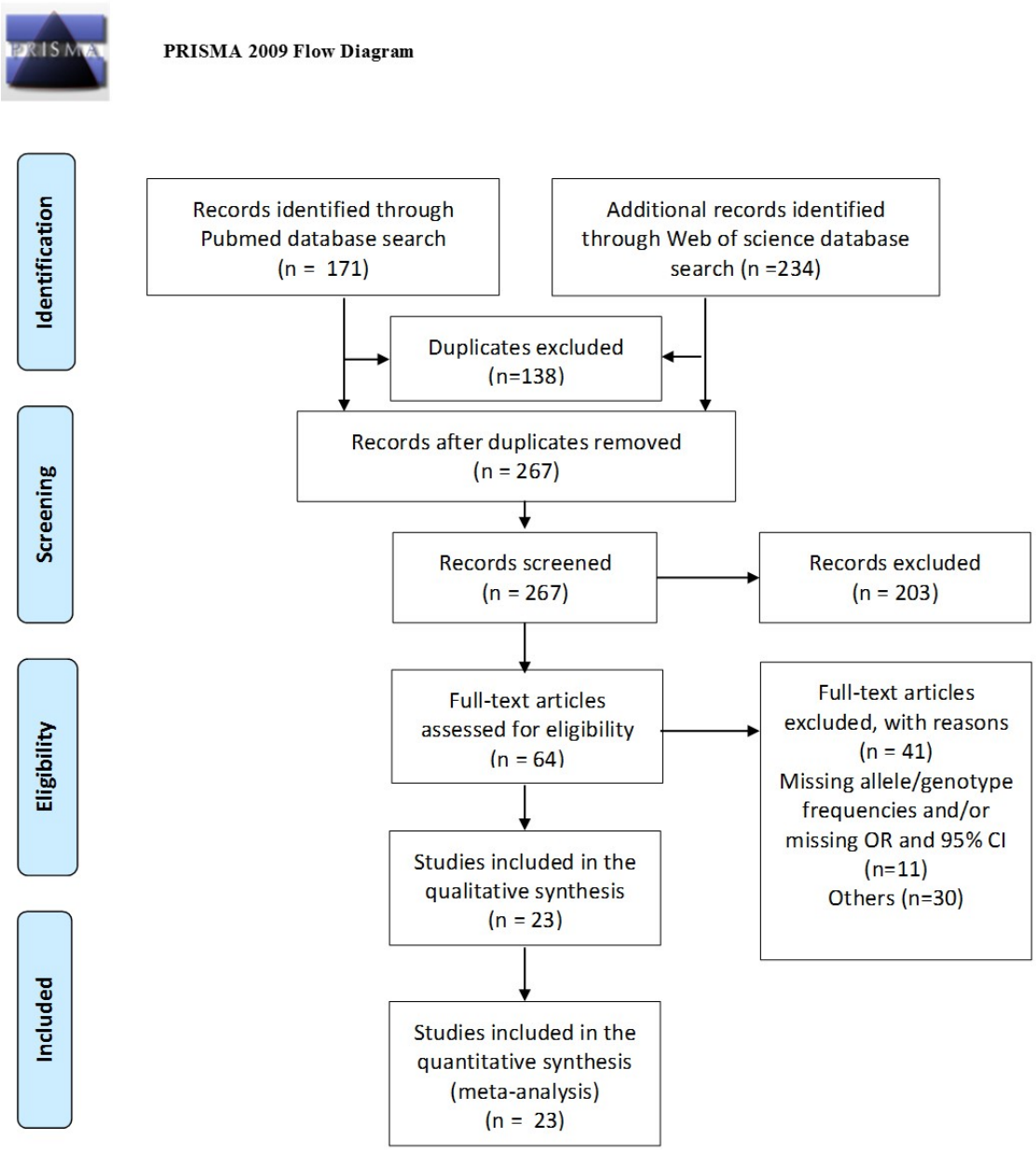


Supplementary Figure S2: Forest plots of the association between *DAO* polymorphisms and schizophrenia in the allele model. Forest plots of all studies, only Caucasian and only Asian samples showing association between *DAO* rs2111902 (**A**), rs3918346 (**B**), rs3918347 (**C**), rs3741775 (**D**), rs3825251 (**E**), rs2070586 (**F**), and rs2070587 (**G**) polymorphisms and schizophrenia; *$p<0.05$ (significant). There was no significant association found between above mentioned *DAO* polymorphisms and schizophrenia.

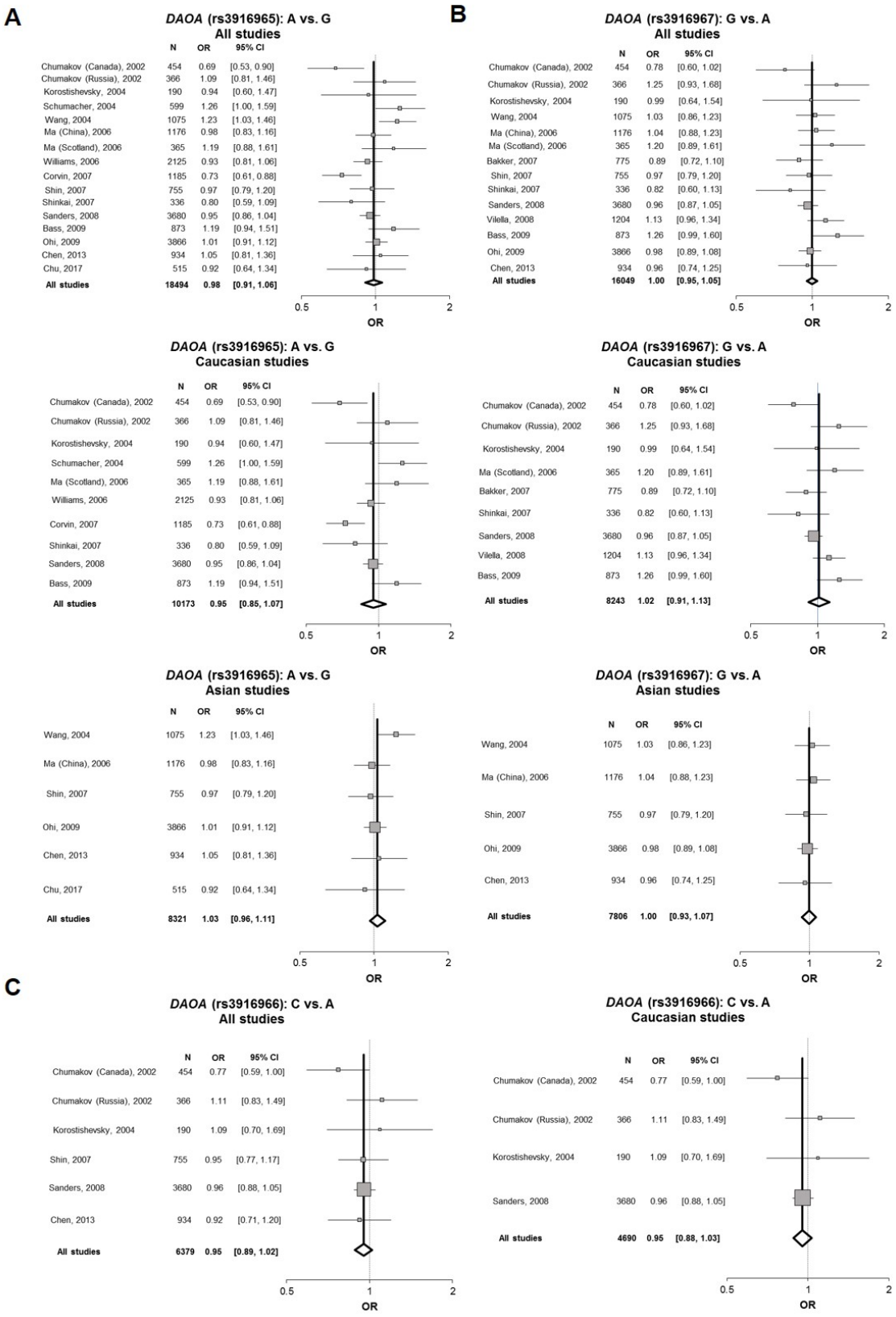

A
DAO (rs2111902): G vs. T
All studies
I²=65%, p=0.0003*
DAO (rs2111902): G vs. T
Caucasian studies
I²=75%, p=0.00001*
DAO (rs2111902): G vs. T
Asian studies
I²=11%, p=0.34
B
DAO (rs3918346): T vs. C
All studies
I²=74%, p=0.00001*
DAO (rs3918346): T vs. C
Caucasian studies
I²=81%, p=0.00001*
DAO (rs3918346): T vs. C
Asian studies
I²=37%, p=0.19
C
DAO (rs3741775): G vs. T
All studies
I²=75%, p=0.00001*
DAO (rs3741775): G vs. T
Caucasian studies
I²=67%, p=0.004*
DAO (rs3741775): G vs. T
Asian studies
I²=86%, p=0.00001*
D
DAO (rs3918347): G vs. A
All studies
I²=65%, p=0.02*
DAO (rs3918347): G vs. A
Caucasian studies
I²=71%, p=0.03
E
DAO (rs4623951): C vs. T
All studies
I²=0%, p=0.83
F
DAO (rs3825251): G vs. A
All studies
I²=37%, p=0.15
DAO (rs3825251): G vs. A
Caucasian studies
I²=77%, p=0.01*
DAO (rs3825251): G vs. A
Asian studies
I²=0%, p=0.87
Precision
OR



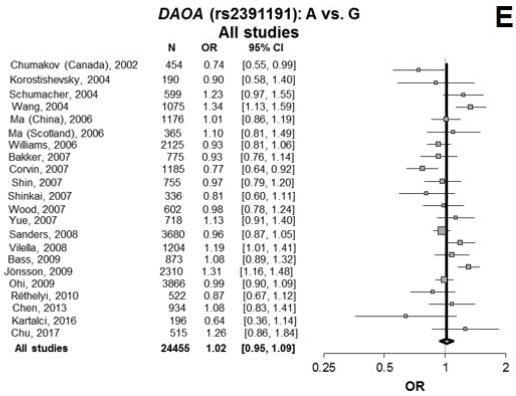
Supplementary Figure S3: Heterogeneity funnel plots of *DAO* rs2111902 (**A**), rs3918346 (**B**), rs3741775 (**C**), rs3918347 (**D**), rs4623951 (**E**), rs3825251 (**F**), rs2070586 (**G**), and rs2070587 (**H**) polymorphisms in all, only Caucasian and only Asian studies. The heterogeneity between studies was assessed by inconsistency index (I^2), and was considered statistically significant if $p<0.05$ (*).

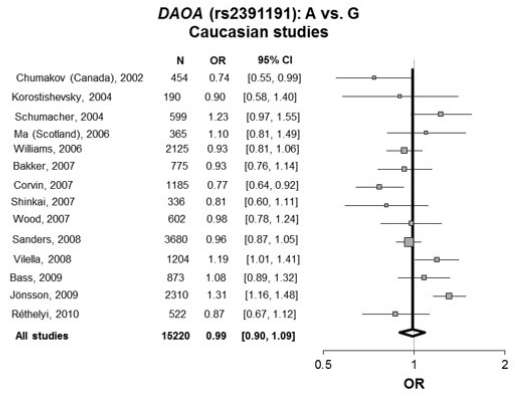


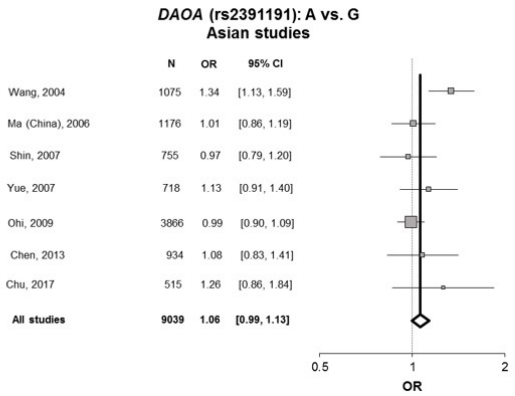
Supplementary Figure S4: PRISMA Flow diagram for literature search of *DAOA* SNP data published till March 21, 2017. Search terms were (DAOA OR G72) AND SCHIZOPHRENIA. OR: odds ratio; CI: confidence interval.


A
DAOA (rs3916965): A vs. G
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.69 [0.53, 0.90]
Chumakov (Russia), 2002 366 1.09 [0.81, 1.46]
Korostishevsky, 2004 190 0.94 [0.60, 1.47]
Schumacher, 2004 599 1.26 [1.00, 1.59]
Wang, 2004 1075 1.23 [1.03, 1.46]
Ma (China), 2006 1176 0.98 [0.83, 1.16]
Ma (Scotland), 2006 365 1.19 [0.88, 1.61]
Williams, 2006 2125 0.93 [0.81, 1.06]
Corvin, 2007 1185 0.73 [0.61, 0.88]
Shin, 2007 755 0.97 [0.79, 1.20]
Shinkai, 2007 336 0.80 [0.59, 1.09]
Sanders, 2008 3680 0.95 [0.86, 1.04]
Bass, 2009 873 1.19 [0.94, 1.51]
Ohi, 2009 3866 1.01 [0.91, 1.12]
Chen, 2013 934 1.05 [0.81, 1.36]
Chu, 2017 515 0.92 [0.64, 1.34]
All studies 18494 0.98 [0.91, 1.06]
0.5 1 2
OR
B
DAOA (rs3916967): G vs. A
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.78 [0.60, 1.02]
Chumakov (Russia), 2002 366 1.25 [0.93, 1.68]
Korostishevsky, 2004 190 0.99 [0.64, 1.54]
Wang, 2004 1075 1.03 [0.86, 1.23]
Ma (China), 2006 1176 1.04 [0.88, 1.23]
Ma (Scotland), 2006 365 1.20 [0.89, 1.61]
Bakker, 2007 775 0.89 [0.72, 1.10]
Shin, 2007 755 0.97 [0.79, 1.20]
Shinkai, 2007 336 0.82 [0.60, 1.13]
Sanders, 2008 3680 0.96 [0.87, 1.05]
Vilella, 2008 1204 1.13 [0.96, 1.34]
Bass, 2009 873 1.26 [0.99, 1.60]
Ohi, 2009 3866 0.98 [0.89, 1.08]
Chen, 2013 934 0.96 [0.74, 1.25]
All studies 16049 1.00 [0.95, 1.05]
0.5 1 2
OR
DAOA (rs3916965): A vs. G
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.69 [0.53, 0.90]
Chumakov (Russia), 2002 366 1.09 [0.81, 1.46]
Korostishevsky, 2004 190 0.94 [0.60, 1.47]
Schumacher, 2004 599 1.26 [1.00, 1.59]
Ma (Scotland), 2006 365 1.19 [0.88, 1.61]
Williams, 2006 2125 0.93 [0.81, 1.06]
Corvin, 2007 1185 0.73 [0.61, 0.88]
Shinkai, 2007 336 0.80 [0.59, 1.09]
Sanders, 2008 3680 0.95 [0.86, 1.04]
Bass, 2009 873 1.19 [0.94, 1.51]
All studies 10173 0.95 [0.85, 1.07]
0.5 1 2
OR
DAOA (rs3916967): G vs. A
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.78 [0.60, 1.02]
Chumakov (Russia), 2002 366 1.25 [0.93, 1.68]
Korostishevsky, 2004 190 0.99 [0.64, 1.54]
Ma (Scotland), 2006 365 1.20 [0.89, 1.61]
Bakker, 2007 775 0.89 [0.72, 1.10]
Shinkai, 2007 336 0.82 [0.60, 1.13]
Sanders, 2008 3680 0.96 [0.87, 1.05]
Vilella, 2008 1204 1.13 [0.96, 1.34]
Bass, 2009 873 1.26 [0.99, 1.60]
All studies 8243 1.02 [0.91, 1.13]
0.5 1 2
OR
DAOA (rs3916965): A vs. G
Asian studies
N OR 95% CI
Wang, 2004 1075 1.23 [1.03, 1.46]
Ma (China), 2006 1176 0.98 [0.83, 1.16]
Shin, 2007 755 0.97 [0.79, 1.20]
Ohi, 2009 3866 1.01 [0.91, 1.12]
Chen, 2013 934 1.05 [0.81, 1.36]
Chu, 2017 515 0.92 [0.64, 1.34]
All studies 8321 1.03 [0.96, 1.11]
0.5 1 2
OR
DAOA (rs3916967): G vs. A
Asian studies
N OR 95% CI
Wang, 2004 1075 1.03 [0.86, 1.23]
Ma (China), 2006 1176 1.04 [0.88, 1.23]
Shin, 2007 755 0.97 [0.79, 1.20]
Ohi, 2009 3866 0.98 [0.89, 1.08]
Chen, 2013 934 0.96 [0.74, 1.25]
All studies 7806 1.00 [0.93, 1.07]
0.5 1 2
OR
C
DAOA (rs3916966): C vs. A
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.77 [0.59, 1.00]
Chumakov (Russia), 2002 366 1.11 [0.83, 1.49]
Korostishevsky, 2004 190 1.09 [0.70, 1.69]
Shin, 2007 755 0.95 [0.77, 1.17]
Sanders, 2008 3680 0.96 [0.88, 1.05]
Chen, 2013 934 0.92 [0.71, 1.20]
All studies 6379 0.95 [0.89, 1.02]
0.5 1 2
OR
DAOA (rs3916966): C vs. A
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.77 [0.59, 1.00]
Chumakov (Russia), 2002 366 1.11 [0.83, 1.49]
Korostishevsky, 2004 190 1.09 [0.70, 1.69]
Sanders, 2008 3680 0.96 [0.88, 1.05]
All studies 4690 0.95 [0.88, 1.03]
0.5 1 2
OR

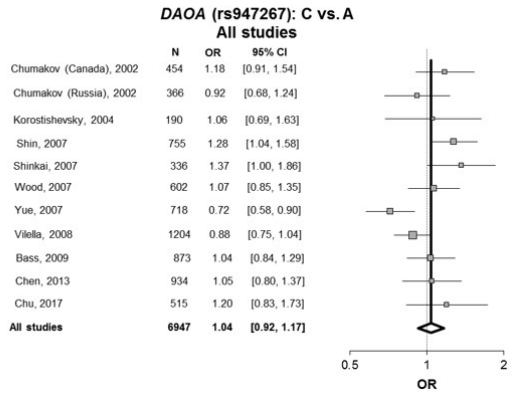
D

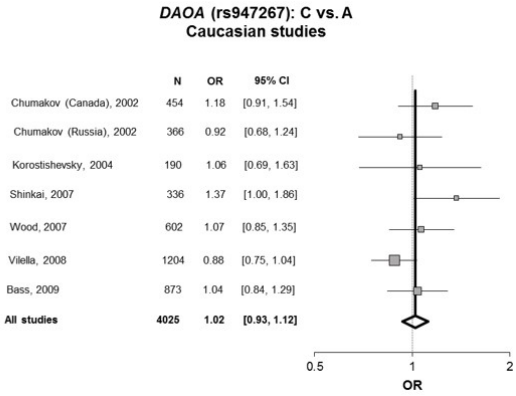

DAOA (rs2391191): A vs. G
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.74 [0.55, 0.99]
Korostishevsky, 2004 190 0.90 [0.58, 1.40]
Schumacher, 2004 599 1.23 [0.97, 1.55]
Wang, 2004 1075 1.34 [1.13, 1.59]
Ma (China), 2006 1176 1.01 [0.86, 1.19]
Ma (Scotland), 2006 365 1.10 [0.81, 1.49]
Williams, 2006 2125 0.93 [0.81, 1.06]
Bakker, 2007 775 0.93 [0.76, 1.14]
Corvin, 2007 1185 0.77 [0.64, 0.92]
Shin, 2007 755 0.97 [0.79, 1.20]
Shinkai, 2007 336 0.81 [0.60, 1.11]
Wood, 2007 602 0.98 [0.78, 1.24]
Yue, 2007 718 1.13 [0.91, 1.40]
Sanders, 2008 3680 0.96 [0.87, 1.05]
Vilella, 2008 1204 1.19 [1.01, 1.41]
Bass, 2009 873 1.08 [0.89, 1.32]
Jönsson, 2009 2310 1.31 [1.16, 1.48]
Ohi, 2009 3866 0.99 [0.90, 1.09]
Réthelyi, 2010 522 0.87 [0.67, 1.12]
Chen, 2013 934 1.08 [0.83, 1.41]
Kartalci, 2016 196 0.64 [0.36, 1.14]
Chu, 2017 515 1.26 [0.86, 1.84]
All studies 24455 1.02 [0.95, 1.09]
0.25 0.5 1 2
OR

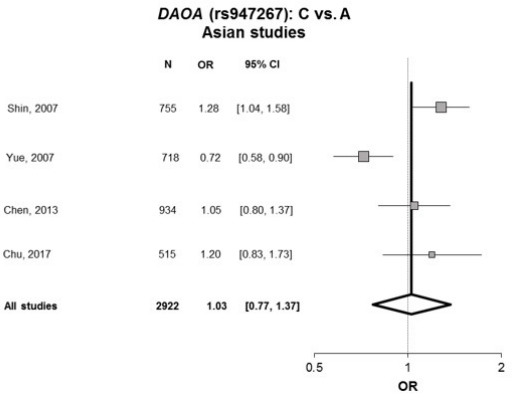

DAOA (rs2391191): A vs. G
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 0.74 [0.55, 0.99]
Korostishevsky, 2004 190 0.90 [0.58, 1.40]
Schumacher, 2004 599 1.23 [0.97, 1.55]
Ma (Scotland), 2006 365 1.10 [0.81, 1.49]
Williams, 2006 2125 0.93 [0.81, 1.06]
Bakker, 2007 775 0.93 [0.76, 1.14]
Corvin, 2007 1185 0.77 [0.64, 0.92]
Shinkai, 2007 336 0.81 [0.60, 1.11]
Wood, 2007 602 0.98 [0.78, 1.24]
Sanders, 2008 3680 0.96 [0.87, 1.05]
Vilella, 2008 1204 1.19 [1.01, 1.41]
Bass, 2009 873 1.08 [0.89, 1.32]
Jönsson, 2009 2310 1.31 [1.16, 1.48]
Réthelyi, 2010 522 0.87 [0.67, 1.12]
All studies 15220 0.99 [0.90, 1.09]
0.5 1 2
OR


DAOA (rs2391191): A vs. G
Asian studies
N OR 95% CI
Wang, 2004 1075 1.34 [1.13, 1.59]
Ma (China), 2006 1176 1.01 [0.86, 1.19]
Shin, 2007 755 0.97 [0.79, 1.20]
Yue, 2007 718 1.13 [0.91, 1.40]
Ohi, 2009 3866 0.99 [0.90, 1.09]
Chen, 2013 934 1.08 [0.83, 1.41]
Chu, 2017 515 1.26 [0.86, 1.84]
All studies 9039 1.06 [0.99, 1.13]
0.5 1 2
OR

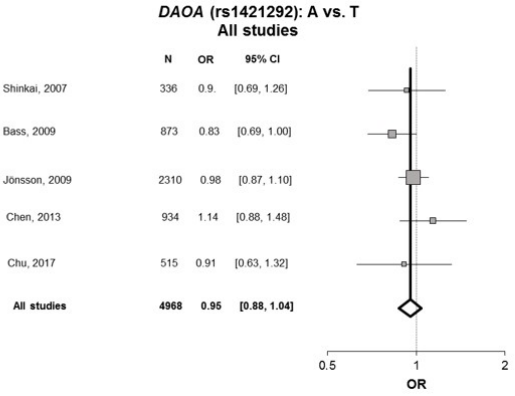
E

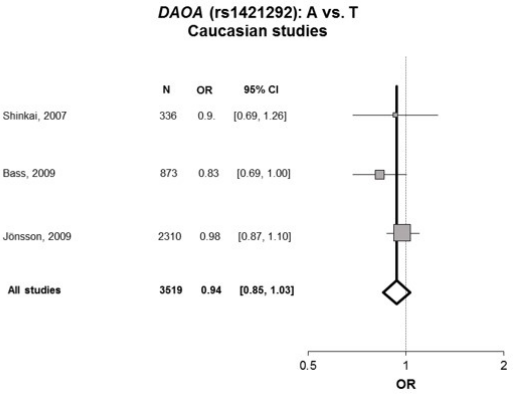

DAOA (rs947267): C vs. A
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 1.18 [0.91, 1.54]
Chumakov (Russia), 2002 366 0.92 [0.68, 1.24]
Korostishevsky, 2004 190 1.06 [0.69, 1.63]
Shin, 2007 755 1.28 [1.04, 1.58]
Shinkai, 2007 336 1.37 [1.00, 1.86]
Wood, 2007 602 1.07 [0.85, 1.35]
Yue, 2007 718 0.72 [0.58, 0.90]
Vilella, 2008 1204 0.88 [0.75, 1.04]
Bass, 2009 873 1.04 [0.84, 1.29]
Chen, 2013 934 1.05 [0.80, 1.37]
Chu, 2017 515 1.20 [0.83, 1.73]
All studies 6947 1.04 [0.92, 1.17]
0.5 1 2
OR


DAOA (rs947267): C vs. A
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 1.18 [0.91, 1.54]
Chumakov (Russia), 2002 366 0.92 [0.68, 1.24]
Korostishevsky, 2004 190 1.06 [0.69, 1.63]
Shinkai, 2007 336 1.37 [1.00, 1.86]
Wood, 2007 602 1.07 [0.85, 1.35]
Vilella, 2008 1204 0.88 [0.75, 1.04]
Bass, 2009 873 1.04 [0.84, 1.29]
All studies 4025 1.02 [0.93, 1.12]
0.5 1 2
OR

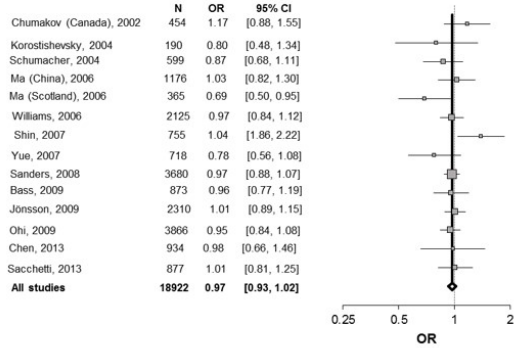

DAOA (rs947267): C vs. A
Asian studies
N OR 95% CI
Shin, 2007 755 1.28 [1.04, 1.58]
Yue, 2007 718 0.72 [0.58, 0.90]
Chen, 2013 934 1.05 [0.80, 1.37]
Chu, 2017 515 1.20 [0.83, 1.73]
All studies 2922 1.03 [0.77, 1.37]
0.5 1 2
OR

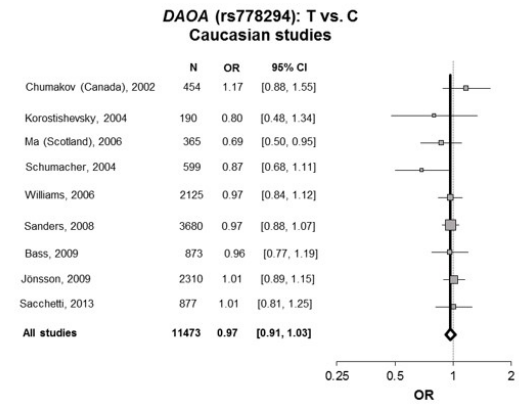
F

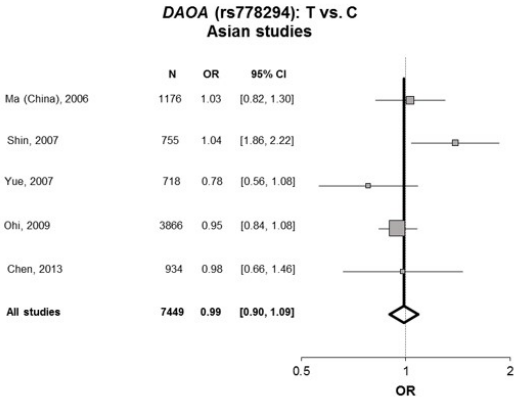

DAOA (rs1421292): A vs. T
All studies
N OR 95% CI
Shinkai, 2007 336 0.9. [0.69, 1.26]
Bass, 2009 873 0.83 [0.69, 1.00]
Jönsson, 2009 2310 0.98 [0.87, 1.10]
Chen, 2013 934 1.14 [0.88, 1.48]
Chu, 2017 515 0.91 [0.63, 1.32]
All studies 4968 0.95 [0.88, 1.04]
0.5 1 2
OR


DAOA (rs1421292): A vs. T
Caucasian studies
N OR 95% CI
Shinkai, 2007 336 0.9. [0.69, 1.26]
Bass, 2009 873 0.83 [0.69, 1.00]
Jönsson, 2009 2310 0.98 [0.87, 1.10]
All studies 3519 0.94 [0.85, 1.03]
0.5 1 2
OR

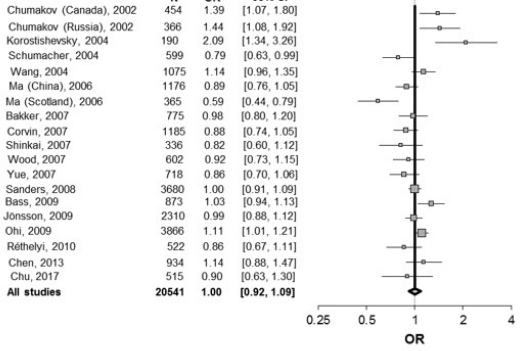
G

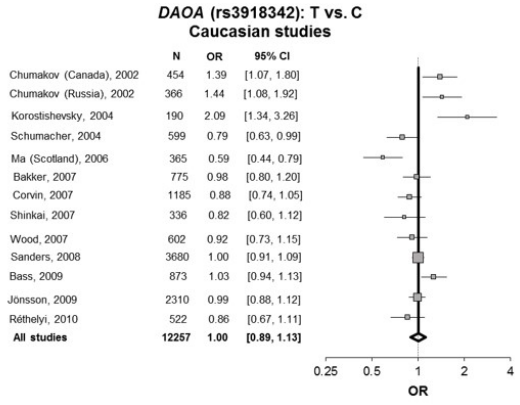

DAOA (rs778294): T vs. C
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 1.17 [0.88, 1.55]
Korostishevsky, 2004 190 0.80 [0.48, 1.34]
Schumacher, 2004 599 0.87 [0.68, 1.11]
Ma (China), 2006 1176 1.03 [0.82, 1.30]
Ma (Scotland), 2006 365 0.69 [0.50, 0.95]
Williams, 2006 2125 0.97 [0.84, 1.12]
Shin, 2007 755 1.04 [1.86, 2.22]
Yue, 2007 718 0.78 [0.56, 1.08]
Sanders, 2008 3680 0.97 [0.88, 1.07]
Bass, 2009 873 0.96 [0.77, 1.19]
Jönsson, 2009 2310 1.01 [0.89, 1.15]
Ohi, 2009 3866 0.95 [0.84, 1.08]
Chen, 2013 934 0.98 [0.66, 1.46]
Sacchetti, 2013 877 1.01 [0.81, 1.25]
All studies 18922 0.97 [0.93, 1.02]
0.25 0.5 1 2
OR

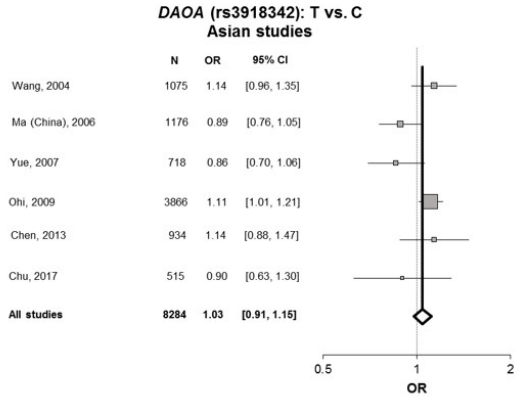

DAOA (rs778294): T vs. C
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 1.17 [0.88, 1.55]
Korostishevsky, 2004 190 0.80 [0.48, 1.34]
Ma (Scotland), 2006 365 0.69 [0.50, 0.95]
Schumacher, 2004 599 0.87 [0.68, 1.11]
Williams, 2006 2125 0.97 [0.84, 1.12]
Sanders, 2008 3680 0.97 [0.88, 1.07]
Bass, 2009 873 0.96 [0.77, 1.19]
Jönsson, 2009 2310 1.01 [0.89, 1.15]
Sacchetti, 2013 877 1.01 [0.81, 1.25]
All studies 11473 0.97 [0.91, 1.03]
0.25 0.5 1 2
OR

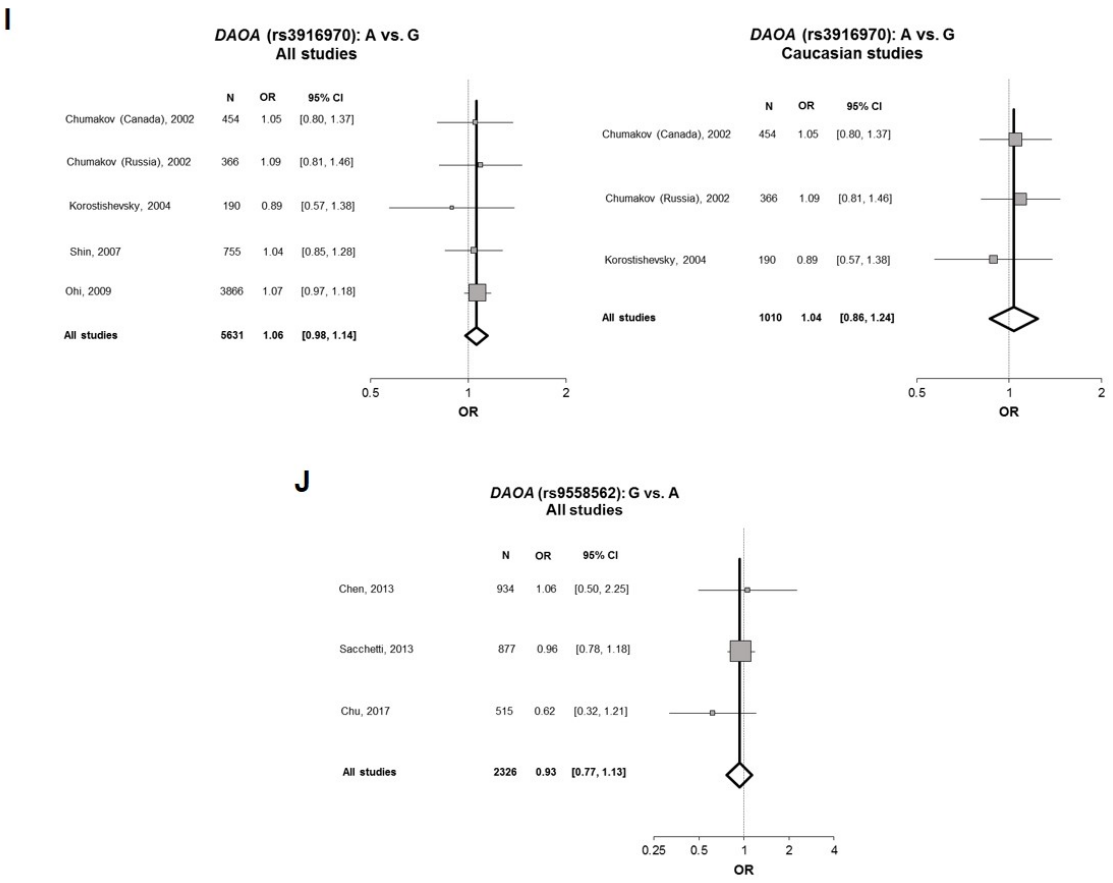

DAOA (rs778294): T vs. C
Asian studies
N OR 95% CI
Ma (China), 2006 1176 1.03 [0.82, 1.30]
Shin, 2007 755 1.04 [1.86, 2.22]
Yue, 2007 718 0.78 [0.56, 1.08]
Ohi, 2009 3866 0.95 [0.84, 1.08]
Chen, 2013 934 0.98 [0.66, 1.46]
All studies 7449 0.99 [0.90, 1.09]
0.5 1 2
OR

H

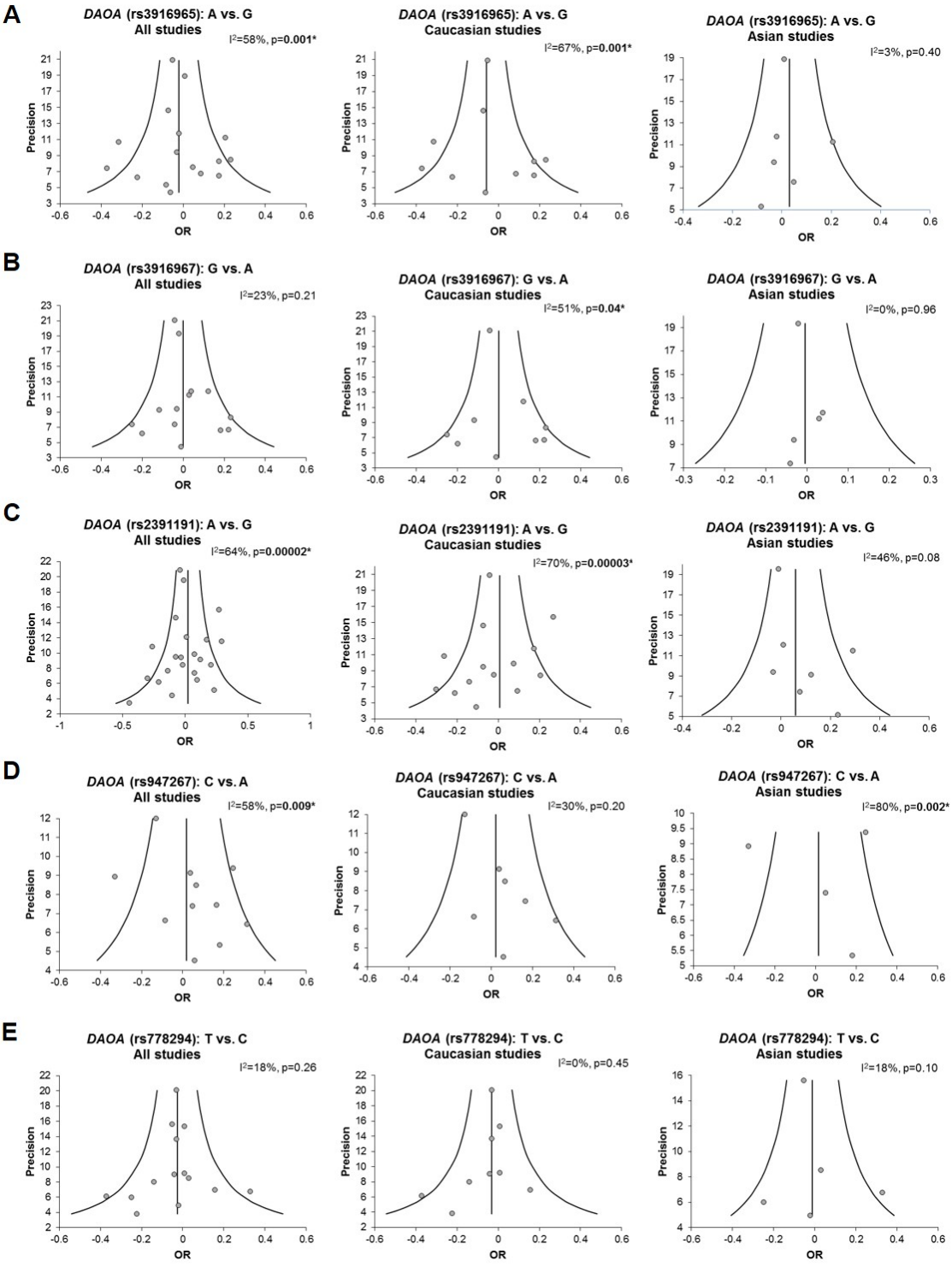

DAOA (rs3918342): T vs. C
All studies
N OR 95% CI
Chumakov (Canada), 2002 454 1.39 [1.07, 1.80]
Chumakov (Russia), 2002 366 1.44 [1.08, 1.92]
Korostishevsky, 2004 190 2.09 [1.34, 3.26]
Schumacher, 2004 599 0.79 [0.63, 0.99]
Wang, 2004 1075 1.14 [0.96, 1.35]
Ma (China), 2006 1176 0.89 [0.76, 1.05]
Ma (Scotland), 2006 365 0.59 [0.44, 0.79]
Bakker, 2007 775 0.98 [0.80, 1.20]
Corvin, 2007 1185 0.88 [0.74, 1.05]
Shinkai, 2007 336 0.82 [0.60, 1.12]
Wood, 2007 602 0.92 [0.73, 1.15]
Yue, 2007 718 0.86 [0.70, 1.06]
Sanders, 2008 3680 1.00 [0.91, 1.09]
Bass, 2009 873 1.03 [0.94, 1.13]
Jönsson, 2009 2310 0.99 [0.88, 1.12]
Ohi, 2009 3866 1.11 [1.01, 1.21]
Réthelyi, 2010 522 0.86 [0.67, 1.11]
Chen, 2013 934 1.14 [0.88, 1.47]
Chu, 2017 515 0.90 [0.63, 1.30]
All studies 20541 1.00 [0.92, 1.09]
0.25 0.5 1 2 4
OR

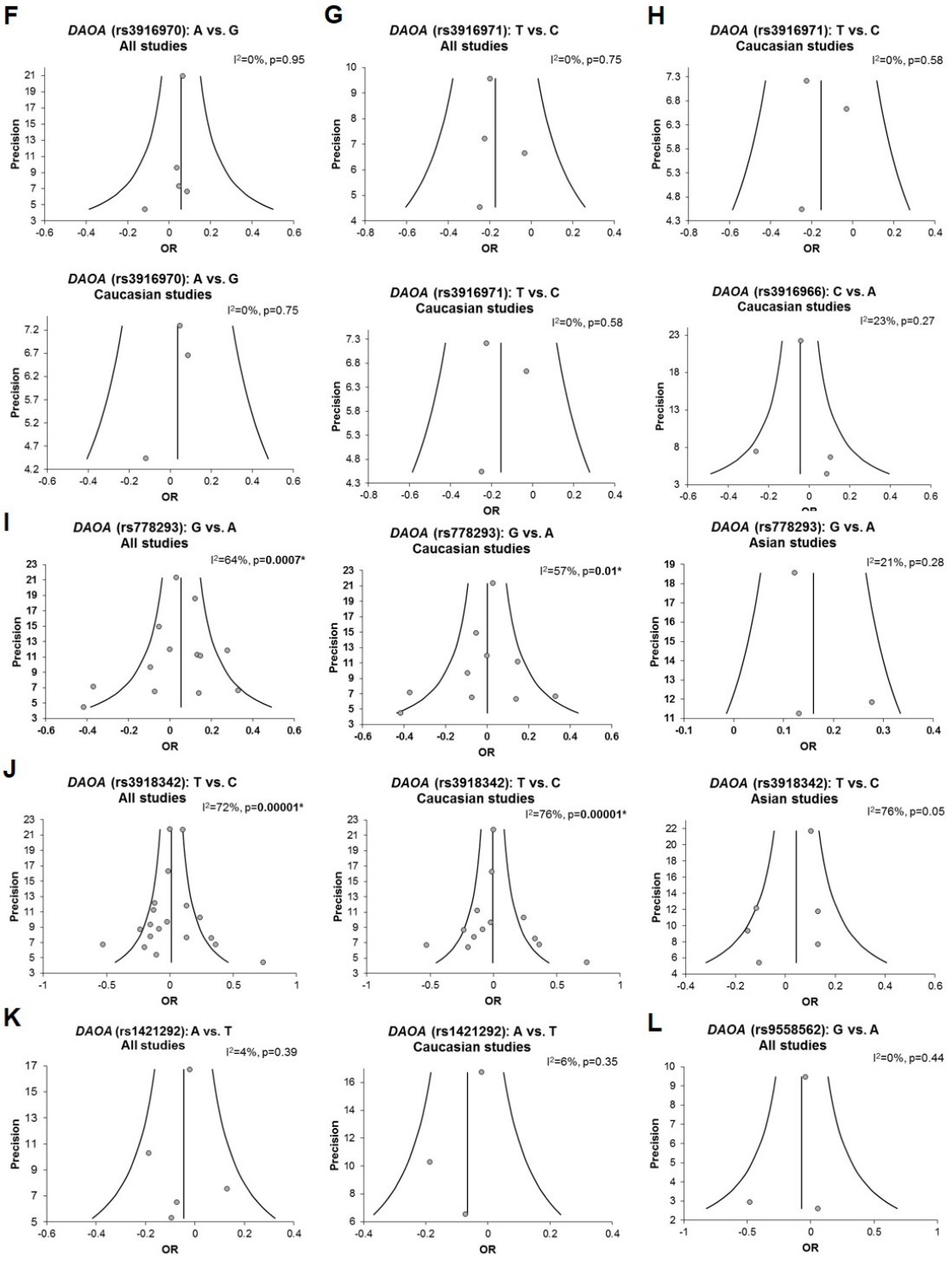

DAOA (rs3918342): T vs. C
Caucasian studies
N OR 95% CI
Chumakov (Canada), 2002 454 1.39 [1.07, 1.80]
Chumakov (Russia), 2002 366 1.44 [1.08, 1.92]
Korostishevsky, 2004 190 2.09 [1.34, 3.26]
Schumacher, 2004 599 0.79 [0.63, 0.99]
Ma (Scotland), 2006 365 0.59 [0.44, 0.79]
Bakker, 2007 775 0.98 [0.80, 1.20]
Corvin, 2007 1185 0.88 [0.74, 1.05]
Shinkai, 2007 336 0.82 [0.60, 1.12]
Wood, 2007 602 0.92 [0.73, 1.15]
Sanders, 2008 3680 1.00 [0.91, 1.09]
Bass, 2009 873 1.03 [0.94, 1.13]
Jönsson, 2009 2310 0.99 [0.88, 1.12]
Réthelyi, 2010 522 0.86 [0.67, 1.11]
All studies 12257 1.00 [0.89, 1.13]
0.25 0.5 1 2 4
OR


DAOA (rs3918342): T vs. C
Asian studies
N OR 95% CI
Wang, 2004 1075 1.14 [0.96, 1.35]
Ma (China), 2006 1176 0.89 [0.76, 1.05]
Yue, 2007 718 0.86 [0.70, 1.06]
Ohi, 2009 3866 1.11 [1.01, 1.21]
Chen, 2013 934 1.14 [0.88, 1.47]
Chu, 2017 515 0.90 [0.63, 1.30]
All studies 8284 1.03 [0.91, 1.15]
0.5 1 2
OR

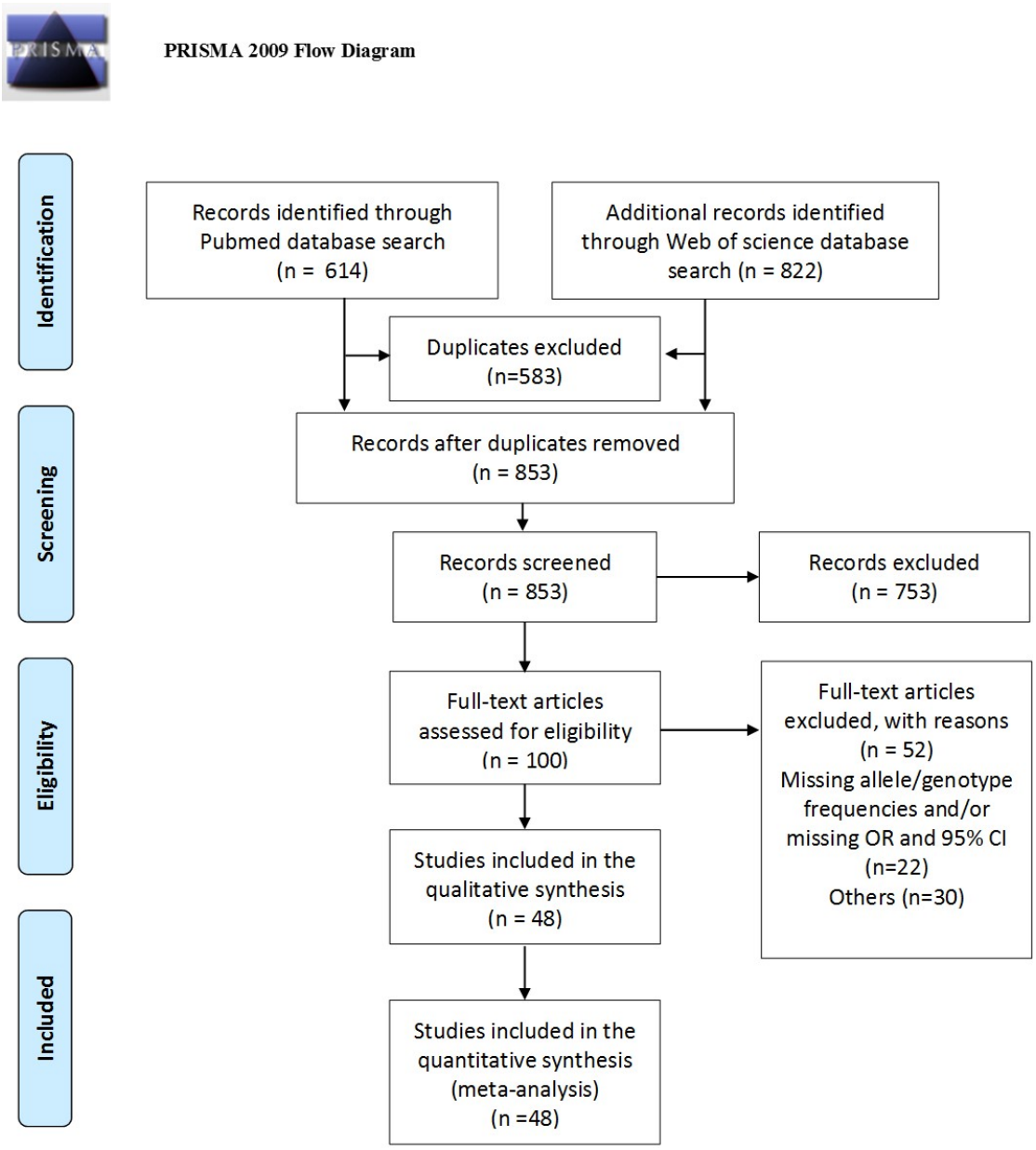


Supplementary Figure S5: Forest plots of the association between *DAOA* polymorphisms and schizophrenia in the allele model. Forest plots of all studies, only Caucasian and only Asian samples showing association between *DAOA* rs3916965 (**A**), rs3916967 (**B**), rs3916966 (**C**), rs2391191 (**D**), rs947267 (**E**), rs1421292 (**F**), rs778294 (**G**), rs3918342 (**H**), rs3916970 (**I**), and rs9558562 (**J**) polymorphisms and schizophrenia; *$p<0.05$ (significant). There was no significant association found between above mentioned *DAOA* polymorphisms and schizophrenia.

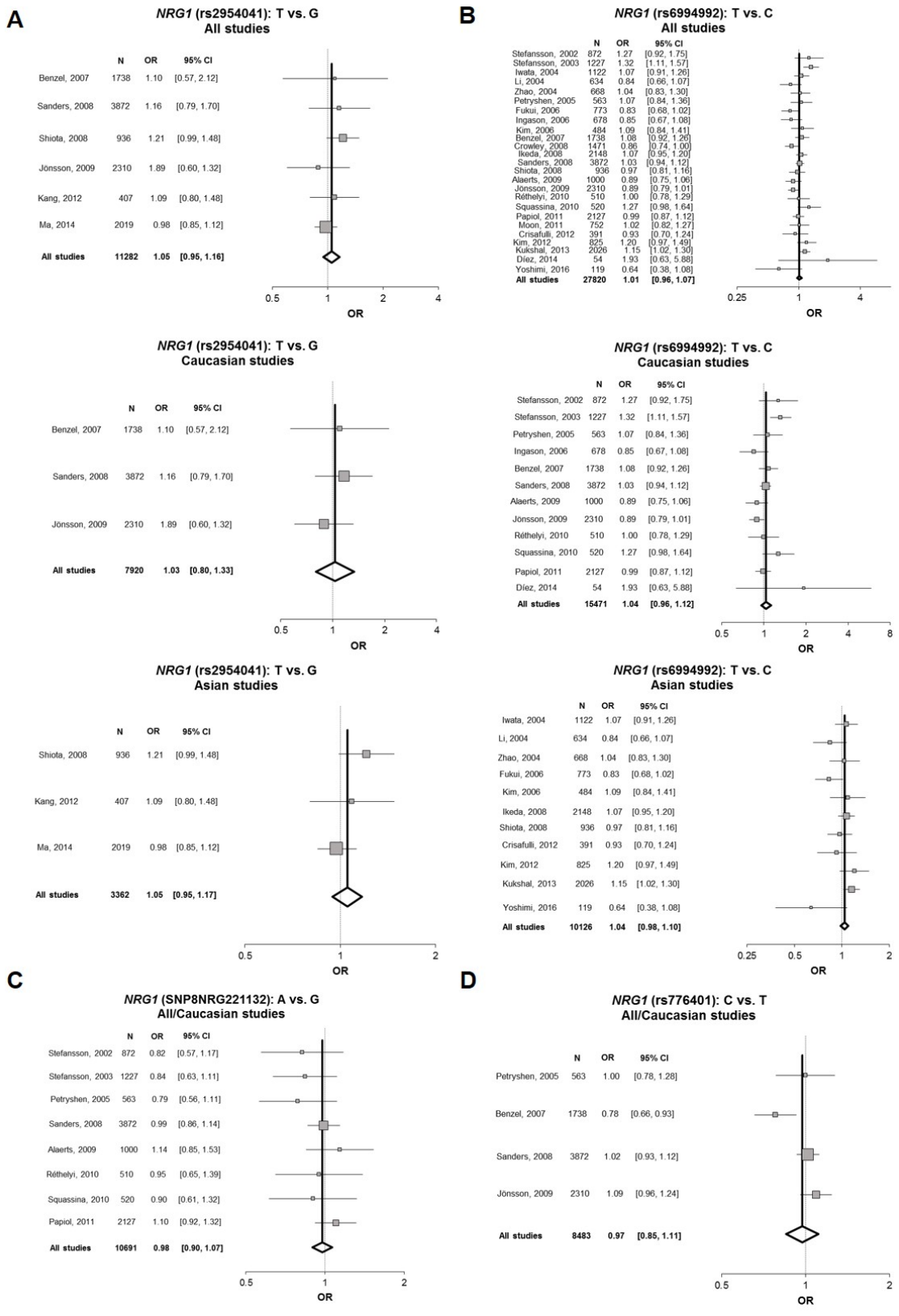

A
DAOA (rs3916965): A vs. G
All studies
I²=58%, p=0.001*
DAOA (rs3916965): A vs. G
Caucasian studies
I²=67%, p=0.001*
DAOA (rs3916965): A vs. G
Asian studies
I²=3%, p=0.40
B
DAOA (rs3916967): G vs. A
All studies
I²=23%, p=0.21
DAOA (rs3916967): G vs. A
Caucasian studies
I²=51%, p=0.04*
DAOA (rs3916967): G vs. A
Asian studies
I²=0%, p=0.96
C
DAOA (rs2391191): A vs. G
All studies
I²=64%, p=0.00002*
DAOA (rs2391191): A vs. G
Caucasian studies
I²=70%, p=0.00003*
DAOA (rs2391191): A vs. G
Asian studies
I²=46%, p=0.08
D
DAOA (rs947267): C vs. A
All studies
I²=58%, p=0.009*
DAOA (rs947267): C vs. A
Caucasian studies
I²=30%, p=0.20
DAOA (rs947267): C vs. A
Asian studies
I²=80%, p=0.002*
E
DAOA (rs778294): T vs. C
All studies
I²=18%, p=0.26
DAOA (rs778294): T vs. C
Caucasian studies
I²=0%, p=0.45
DAOA (rs778294): T vs. C
Asian studies
I²=18%, p=0.10
Precision
OR



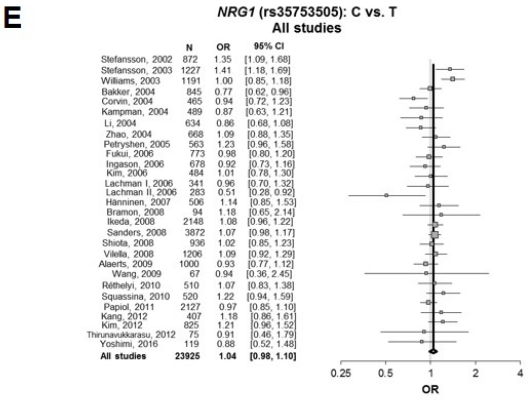
Supplementary Figure S6: Heterogeneity funnel plots of *DAOA* rs3916965 (**A**), rs3916967 (**B**), rs2391191 (**C**), rs947267 (**D**), rs778294 (**E**), rs3916970 (**F**), rs3916971 (**G**), rs3916966 (**H**), rs778293 (**I**), rs3918342 (**J**), rs1421292 (**K**), and rs9558562 (**L**) polymorphisms in all studies, only Caucasian and only Asian samples. The heterogeneity between studies was assessed by inconsistency index (I^2), and was considered statistically significant if $p<0.05$ (*).



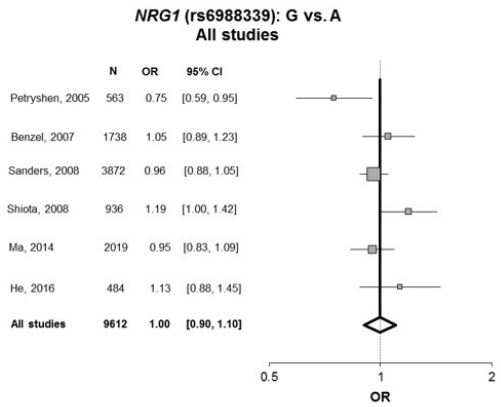
Supplementary Figure S7: PRISMA Flow diagram for literature search of *NRG1* SNP data published till March 21, 2017. Search terms were (NRG1 OR Neuregulin-1 OR Neuregulin 1 OR Neuregulin1) AND SCHIZOPHRENIA. OR: odds ratio; CI: confidence interval.

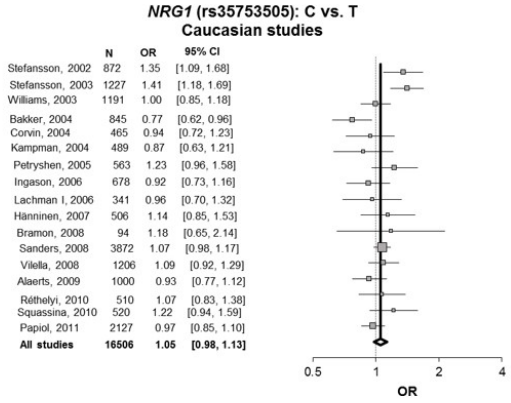

A
NRG1 (rs2954041): T vs. G
All studies
N OR 95% CI
Benzel, 2007 1738 1.10 [0.57, 2.12]
Sanders, 2008 3872 1.16 [0.79, 1.70]
Shiota, 2008 936 1.21 [0.99, 1.48]
Jönsson, 2009 2310 1.89 [0.60, 1.32]
Kang, 2012 407 1.09 [0.80, 1.48]
Ma, 2014 2019 0.98 [0.85, 1.12]
All studies 11282 1.05 [0.95, 1.16]
0.5 1 2 4
OR
B
NRG1 (rs6994992): T vs. C
All studies
N OR 95% CI
Stefansson, 2002 872 1.27 [0.92, 1.75]
Stefansson, 2003 1227 1.32 [1.11, 1.57]
Iwata, 2004 1122 1.07 [0.91, 1.26]
Li, 2004 634 0.84 [0.66, 1.07]
Zhao, 2004 668 1.04 [0.83, 1.30]
Petryshen, 2005 563 1.07 [0.84, 1.36]
Fukui, 2006 773 0.83 [0.68, 1.02]
Ingason, 2006 678 0.85 [0.67, 1.08]
Kim, 2006 484 1.09 [0.84, 1.41]
Benzel, 2007 1738 1.08 [0.92, 1.26]
Crowley, 2008 1471 0.86 [0.74, 1.00]
Ikeda, 2008 2148 1.07 [0.95, 1.20]
Sanders, 2008 3872 1.03 [0.94, 1.12]
Shiota, 2008 936 0.97 [0.81, 1.16]
Alaerts, 2009 1000 0.89 [0.75, 1.06]
Jönsson, 2009 2310 0.89 [0.79, 1.01]
Réthelyi, 2010 510 1.00 [0.78, 1.29]
Squassina, 2010 520 1.27 [0.98, 1.64]
Papiol, 2011 2127 0.99 [0.87, 1.12]
Moon, 2011 752 1.02 [0.82, 1.27]
Crisafulli, 2012 391 0.93 [0.70, 1.24]
Kim, 2012 825 1.20 [0.97, 1.49]
Kukshal, 2013 2026 1.15 [1.02, 1.30]
Díez, 2014 54 1.93 [0.63, 5.88]
Yoshimi, 2016 119 0.64 [0.38, 1.08]
All studies 27820 1.01 [0.96, 1.07]
0.25 1 4
OR
NRG1 (rs2954041): T vs. G
Caucasian studies
N OR 95% CI
Benzel, 2007 1738 1.10 [0.57, 2.12]
Sanders, 2008 3872 1.16 [0.79, 1.70]
Jönsson, 2009 2310 1.89 [0.60, 1.32]
All studies 7920 1.03 [0.80, 1.33]
0.5 1 2 4
OR
NRG1 (rs6994992): T vs. C
Caucasian studies
N OR 95% CI
Stefansson, 2002 872 1.27 [0.92, 1.75]
Stefansson, 2003 1227 1.32 [1.11, 1.57]
Petryshen, 2005 563 1.07 [0.84, 1.36]
Ingason, 2006 678 0.85 [0.67, 1.08]
Benzel, 2007 1738 1.08 [0.92, 1.26]
Sanders, 2008 3872 1.03 [0.94, 1.12]
Alaerts, 2009 1000 0.89 [0.75, 1.06]
Jönsson, 2009 2310 0.89 [0.79, 1.01]
Réthelyi, 2010 510 1.00 [0.78, 1.29]
Squassina, 2010 520 1.27 [0.98, 1.64]
Papiol, 2011 2127 0.99 [0.87, 1.12]
Díez, 2014 54 1.93 [0.63, 5.88]
All studies 15471 1.04 [0.96, 1.12]
0.5 1 2 4 8
OR
NRG1 (rs2954041): T vs. G
Asian studies
N OR 95% CI
Shiota, 2008 936 1.21 [0.99, 1.48]
Kang, 2012 407 1.09 [0.80, 1.48]
Ma, 2014 2019 0.98 [0.85, 1.12]
All studies 3362 1.05 [0.95, 1.17]
0.5 1 2
OR
NRG1 (rs6994992): T vs. C
Asian studies
N OR 95% CI
Iwata, 2004 1122 1.07 [0.91, 1.26]
Li, 2004 634 0.84 [0.66, 1.07]
Zhao, 2004 668 1.04 [0.83, 1.30]
Fukui, 2006 773 0.83 [0.68, 1.02]
Kim, 2006 484 1.09 [0.84, 1.41]
Ikeda, 2008 2148 1.07 [0.95, 1.20]
Shiota, 2008 936 0.97 [0.81, 1.16]
Crisafulli, 2012 391 0.93 [0.70, 1.24]
Kim, 2012 825 1.20 [0.97, 1.49]
Kukshal, 2013 2026 1.15 [1.02, 1.30]
Yoshimi, 2016 119 0.64 [0.38, 1.08]
All studies 10126 1.04 [0.98, 1.10]
0.25 0.5 1 2
OR
C
NRG1 (SNP8NRG221132): A vs. G
All/Caucasian studies
N OR 95% CI
Stefansson, 2002 872 0.82 [0.57, 1.17]
Stefansson, 2003 1227 0.84 [0.63, 1.11]
Petryshen, 2005 563 0.79 [0.56, 1.11]
Sanders, 2008 3872 0.99 [0.86, 1.14]
Alaerts, 2009 1000 1.14 [0.85, 1.53]
Réthelyi, 2010 510 0.95 [0.65, 1.39]
Squassina, 2010 520 0.90 [0.61, 1.32]
Papiol, 2011 2127 1.10 [0.92, 1.32]
All studies 10691 0.98 [0.90, 1.07]
0.5 1 2
OR
D
NRG1 (rs776401): C vs. T
All/Caucasian studies
N OR 95% CI
Petryshen, 2005 563 1.00 [0.78, 1.28]
Benzel, 2007 1738 0.78 [0.66, 0.93]
Sanders, 2008 3872 1.02 [0.93, 1.12]
Jönsson, 2009 2310 1.09 [0.96, 1.24]
All studies 8483 0.97 [0.85, 1.11]
0.5 1 2
OR

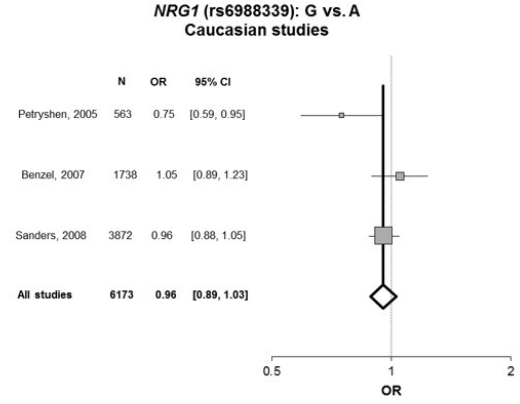
E

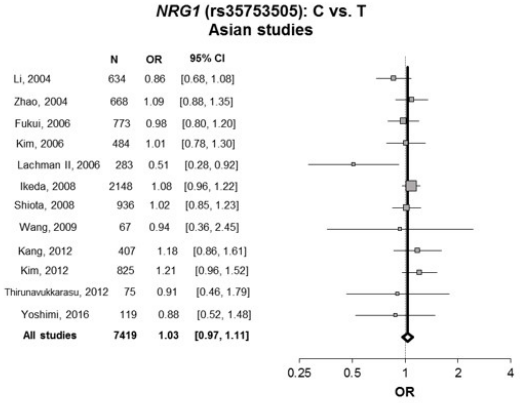

NRG1 (rs35753505): C vs. T
All studies
N OR 95% CI
Stefansson, 2002 872 1.35 [1.09, 1.68]
Stefansson, 2003 1227 1.41 [1.18, 1.69]
Williams, 2003 1191 1.00 [0.85, 1.18]
Bakker, 2004 845 0.77 [0.62, 0.96]
Corvin, 2004 465 0.94 [0.72, 1.23]
Kampman, 2004 489 0.87 [0.63, 1.21]
Li, 2004 634 0.86 [0.68, 1.08]
Zhao, 2004 668 1.09 [0.88, 1.35]
Petryshen, 2005 563 1.23 [0.96, 1.58]
Fukui, 2006 773 0.98 [0.80, 1.20]
Ingason, 2006 678 0.92 [0.73, 1.16]
Kim, 2006 484 1.01 [0.78, 1.30]
Lachman I, 2006 341 0.96 [0.70, 1.32]
Lachman II, 2006 283 0.51 [0.28, 0.92]
Hänninen, 2007 506 1.14 [0.85, 1.53]
Bramon, 2008 94 1.18 [0.65, 2.14]
Ikeda, 2008 2148 1.08 [0.96, 1.22]
Sanders, 2008 3872 1.07 [0.98, 1.17]
Shiota, 2008 936 1.02 [0.85, 1.23]
Vilella, 2008 1206 1.09 [0.92, 1.29]
Alaerts, 2009 1000 0.93 [0.77, 1.12]
Wang, 2009 67 0.94 [0.36, 2.45]
Réthelyi, 2010 510 1.07 [0.83, 1.38]
Squassina, 2010 520 1.22 [0.94, 1.59]
Papiol, 2011 2127 0.97 [0.85, 1.10]
Kang, 2012 407 1.18 [0.86, 1.61]
Kim, 2012 825 1.21 [0.96, 1.52]
Thirunavukkarasu, 2012 75 0.91 [0.46, 1.79]
Yoshimi, 2016 119 0.88 [0.52, 1.48]
All studies 23925 1.04 [0.98, 1.10]
0.25 0.5 1 2 4
OR

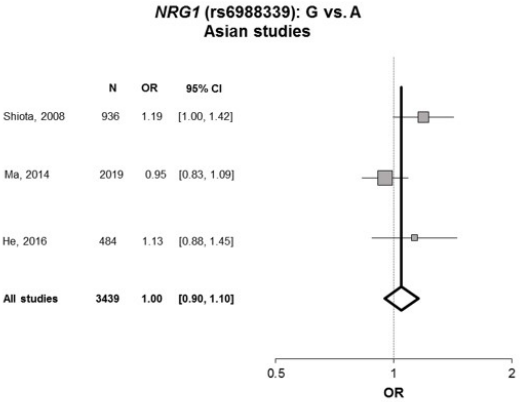
F


NRG1 (rs6988339): G vs. A
All studies
N OR 95% CI
Petryshen, 2005 563 0.75 [0.59, 0.95]
Benzel, 2007 1738 1.05 [0.89, 1.23]
Sanders, 2008 3872 0.96 [0.88, 1.05]
Shiota, 2008 936 1.19 [1.00, 1.42]
Ma, 2014 2019 0.95 [0.83, 1.09]
He, 2016 484 1.13 [0.88, 1.45]
All studies 9612 1.00 [0.90, 1.10]
0.5 1 2
OR

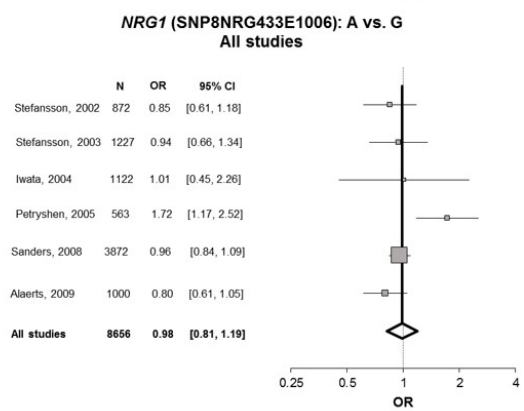

NRG1 (rs35753505): C vs. T
Caucasian studies
N OR 95% CI
Stefansson, 2002 872 1.35 [1.09, 1.68]
Stefansson, 2003 1227 1.41 [1.18, 1.69]
Williams, 2003 1191 1.00 [0.85, 1.18]
Bakker, 2004 845 0.77 [0.62, 0.96]
Corvin, 2004 465 0.94 [0.72, 1.23]
Kampman, 2004 489 0.87 [0.63, 1.21]
Petryshen, 2005 563 1.23 [0.96, 1.58]
Ingason, 2006 678 0.92 [0.73, 1.16]
Lachman I, 2006 341 0.96 [0.70, 1.32]
Hänninen, 2007 506 1.14 [0.85, 1.53]
Bramon, 2008 94 1.18 [0.65, 2.14]
Sanders, 2008 3872 1.07 [0.98, 1.17]
Vilella, 2008 1206 1.09 [0.92, 1.29]
Alaerts, 2009 1000 0.93 [0.77, 1.12]
Réthelyi, 2010 510 1.07 [0.83, 1.38]
Squassina, 2010 520 1.22 [0.94, 1.59]
Papiol, 2011 2127 0.97 [0.85, 1.10]
All studies 16506 1.05 [0.98, 1.13]
0.5 1 2 4
OR

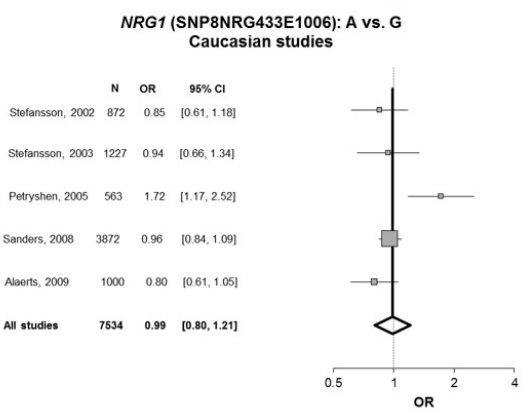

NRG1 (rs6988339): G vs. A
Caucasian studies
N OR 95% CI
Petryshen, 2005 563 0.75 [0.59, 0.95]
Benzel, 2007 1738 1.05 [0.89, 1.23]
Sanders, 2008 3872 0.96 [0.88, 1.05]
All studies 6173 0.96 [0.89, 1.03]
0.5 1 2
OR


NRG1 (rs35753505): C vs. T
Asian studies
N OR 95% CI
Li, 2004 634 0.86 [0.68, 1.08]
Zhao, 2004 668 1.09 [0.88, 1.35]
Fukui, 2006 773 0.98 [0.80, 1.20]
Kim, 2006 484 1.01 [0.78, 1.30]
Lachman II, 2006 283 0.51 [0.28, 0.92]
Ikeda, 2008 2148 1.08 [0.96, 1.22]
Shiota, 2008 936 1.02 [0.85, 1.23]
Wang, 2009 67 0.94 [0.36, 2.45]
Kang, 2012 407 1.18 [0.86, 1.61]
Kim, 2012 825 1.21 [0.96, 1.52]
Thirunavukkarasu, 2012 75 0.91 [0.46, 1.79]
Yoshimi, 2016 119 0.88 [0.52, 1.48]
All studies 7419 1.03 [0.97, 1.11]
0.25 0.5 1 2 4
OR

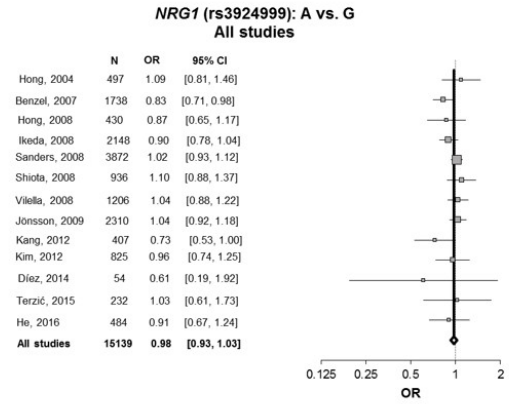

NRG1 (rs6988339): G vs. A
Asian studies
N OR 95% CI
Shiota, 2008 936 1.19 [1.00, 1.42]
Ma, 2014 2019 0.95 [0.83, 1.09]
He, 2016 484 1.13 [0.88, 1.45]
All studies 3439 1.00 [0.90, 1.10]
0.5 1 2
OR

G

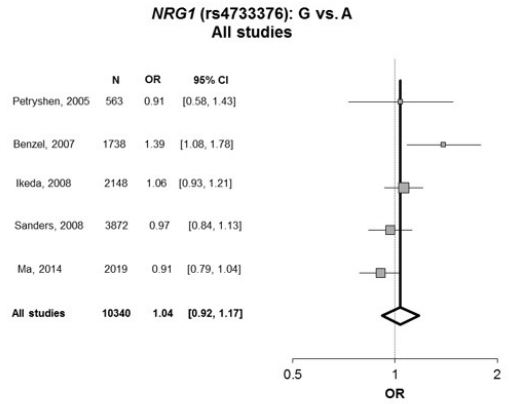

NRG1 (SNP8NRG433E1006): A vs. G
All studies
N OR 95% CI
Stefansson, 2002 872 0.85 [0.61, 1.18]
Stefansson, 2003 1227 0.94 [0.66, 1.34]
Iwata, 2004 1122 1.01 [0.45, 2.26]
Petryshen, 2005 563 1.72 [1.17, 2.52]
Sanders, 2008 3872 0.96 [0.84, 1.09]
Alaerts, 2009 1000 0.80 [0.61, 1.05]
All studies 8656 0.98 [0.81, 1.19]
0.25 0.5 1 2 4
OR

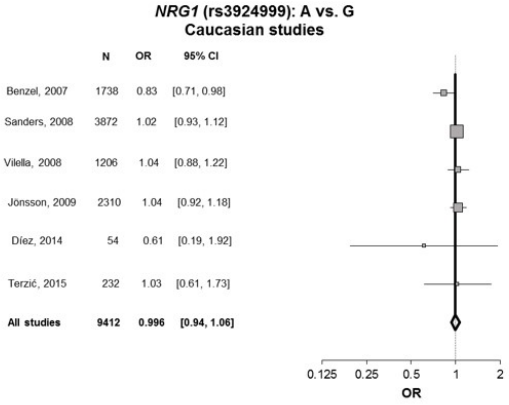

NRG1 (SNP8NRG433E1006): A vs. G
Caucasian studies
N OR 95% CI
Stefansson, 2002 872 0.85 [0.61, 1.18]
Stefansson, 2003 1227 0.94 [0.66, 1.34]
Petryshen, 2005 563 1.72 [1.17, 2.52]
Sanders, 2008 3872 0.96 [0.84, 1.09]
Alaerts, 2009 1000 0.80 [0.61, 1.05]
All studies 7534 0.99 [0.80, 1.21]
0.5 1 2 4
OR

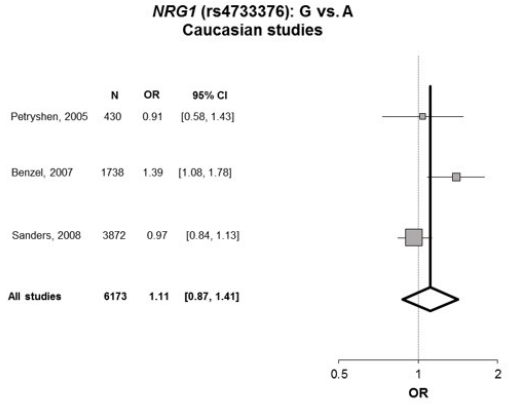
H

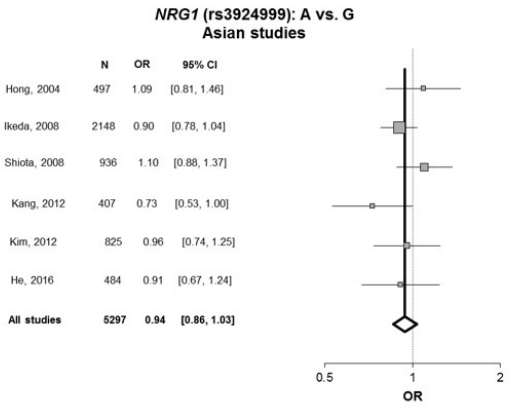

NRG1 (rs3924999): A vs. G
All studies
N OR 95% CI
Hong, 2004 497 1.09 [0.81, 1.46]
Benzel, 2007 1738 0.83 [0.71, 0.98]
Hong, 2008 430 0.87 [0.65, 1.17]
Ikeda, 2008 2148 0.90 [0.78, 1.04]
Sanders, 2008 3872 1.02 [0.93, 1.12]
Shiota, 2008 936 1.10 [0.88, 1.37]
Vilella, 2008 1206 1.04 [0.88, 1.22]
Jönsson, 2009 2310 1.04 [0.92, 1.18]
Kang, 2012 407 0.73 [0.53, 1.00]
Kim, 2012 825 0.96 [0.74, 1.25]
Díez, 2014 54 0.61 [0.19, 1.92]
Terzić, 2015 232 1.03 [0.61, 1.73]
He, 2016 484 0.91 [0.67, 1.24]
All studies 15139 0.98 [0.93, 1.03]
0.125 0.25 0.5 1 2
OR

I

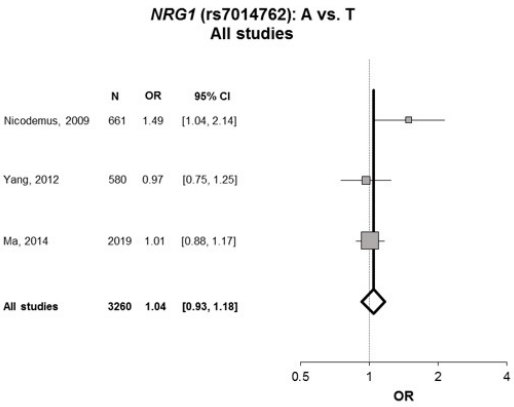

NRG1 (rs4733376): G vs. A
All studies
N OR 95% CI
Petryshen, 2005 563 0.91 [0.58, 1.43]
Benzel, 2007 1738 1.39 [1.08, 1.78]
Ikeda, 2008 2148 1.06 [0.93, 1.21]
Sanders, 2008 3872 0.97 [0.84, 1.13]
Ma, 2014 2019 0.91 [0.79, 1.04]
All studies 10340 1.04 [0.92, 1.17]
0.5 1 2
OR

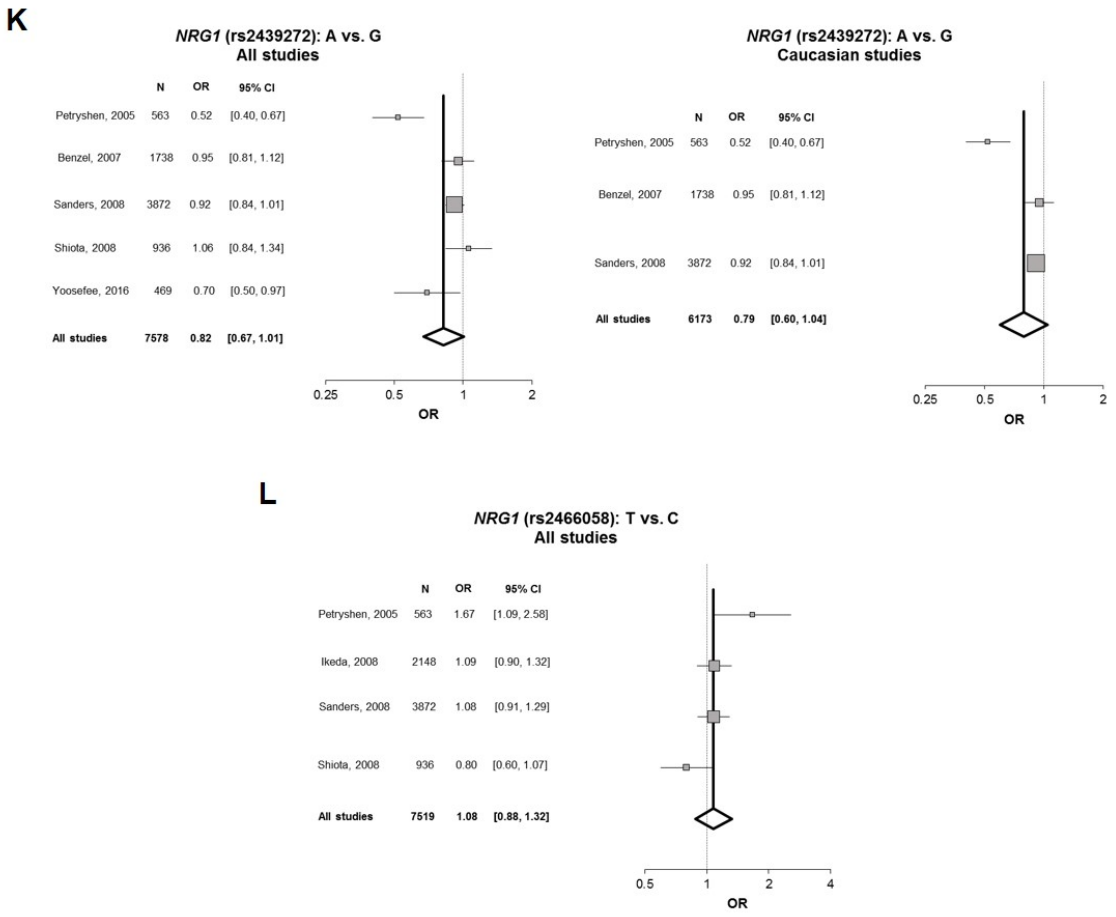

NRG1 (rs3924999): A vs. G
Caucasian studies
N OR 95% CI
Benzel, 2007 1738 0.83 [0.71, 0.98]
Sanders, 2008 3872 1.02 [0.93, 1.12]
Vilella, 2008 1206 1.04 [0.88, 1.22]
Jönsson, 2009 2310 1.04 [0.92, 1.18]
Díez, 2014 54 0.61 [0.19, 1.92]
Terzić, 2015 232 1.03 [0.61, 1.73]
All studies 9412 0.996 [0.94, 1.06]
0.125 0.25 0.5 1 2
OR


NRG1 (rs4733376): G vs. A
Caucasian studies
N OR 95% CI
Petryshen, 2005 430 0.91 [0.58, 1.43]
Benzel, 2007 1738 1.39 [1.08, 1.78]
Sanders, 2008 3872 0.97 [0.84, 1.13]
All studies 6173 1.11 [0.87, 1.41]
0.5 1 2
OR

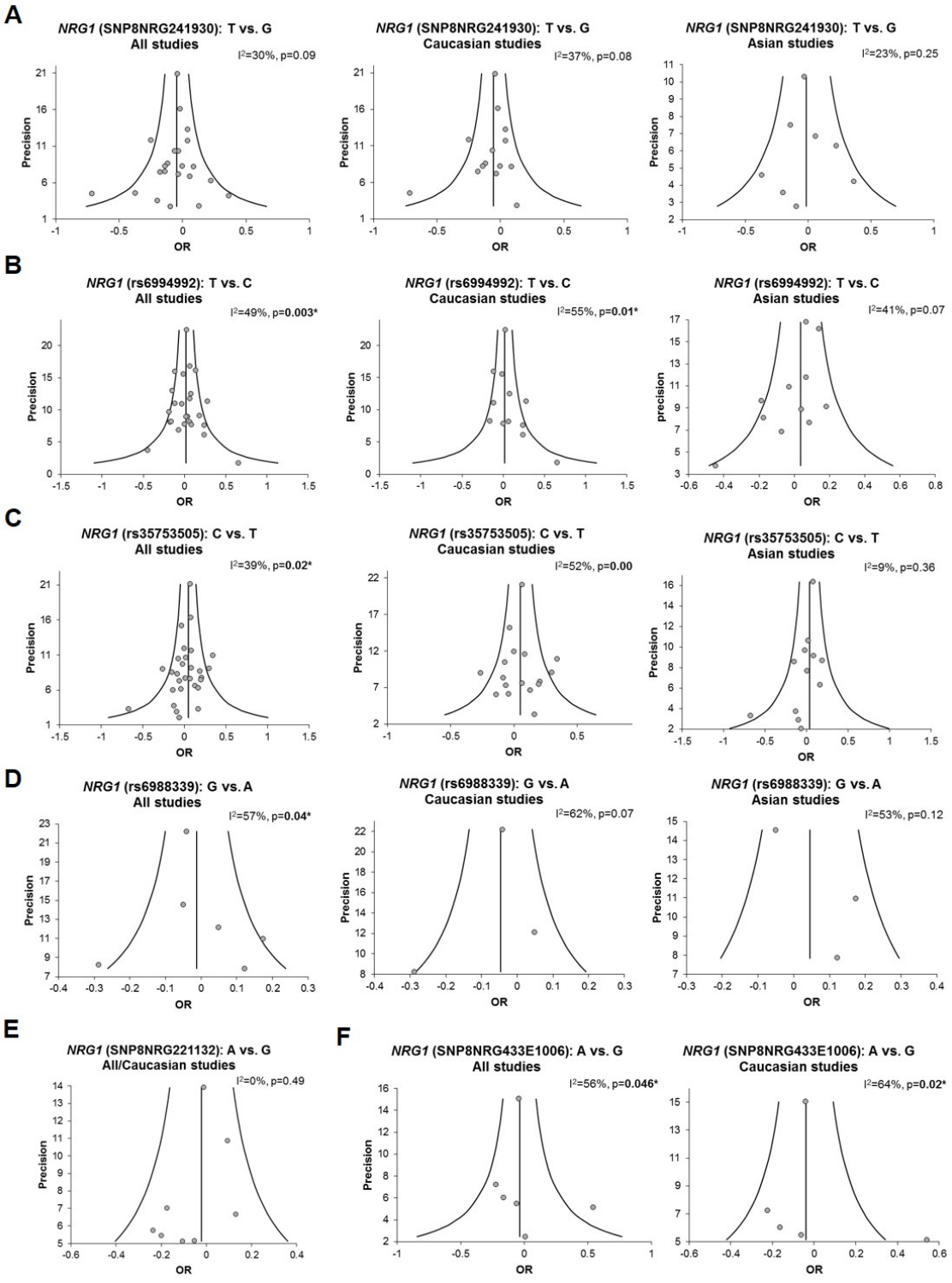

NRG1 (rs3924999): A vs. G
Asian studies
N OR 95% CI
Hong, 2004 497 1.09 [0.81, 1.46]
Ikeda, 2008 2148 0.90 [0.78, 1.04]
Shiota, 2008 936 1.10 [0.88, 1.37]
Kang, 2012 407 0.73 [0.53, 1.00]
Kim, 2012 825 0.96 [0.74, 1.25]
He, 2016 484 0.91 [0.67, 1.24]
All studies 5297 0.94 [0.86, 1.03]
0.5 1 2
OR

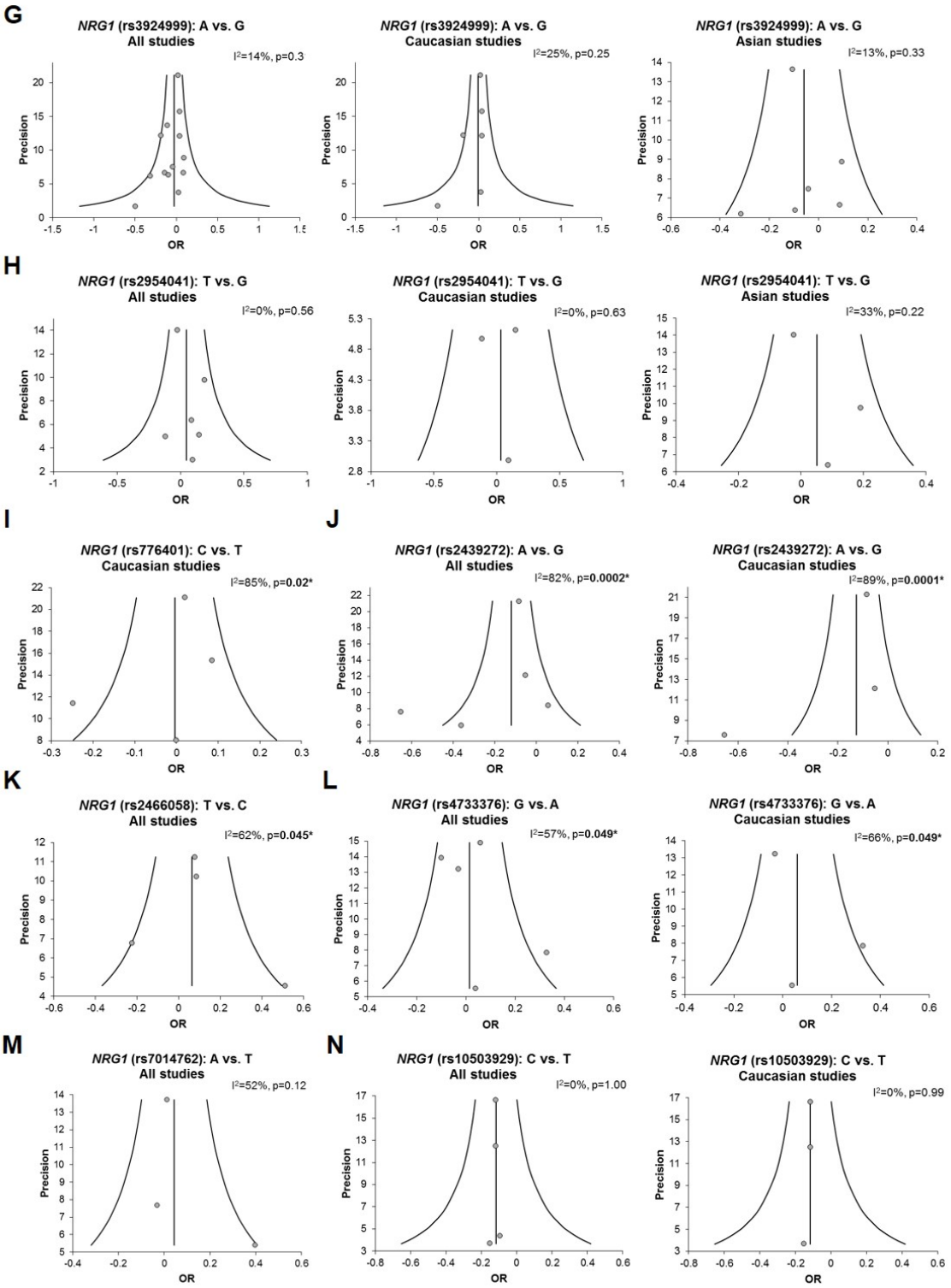
J


NRG1 (rs7014762): A vs. T
All studies
N OR 95% CI
Nicodemus, 2009 661 1.49 [1.04, 2.14]
Yang, 2012 580 0.97 [0.75, 1.25]
Ma, 2014 2019 1.01 [0.88, 1.17]
All studies 3260 1.04 [0.93, 1.18]
0.5 1 2 4
OR

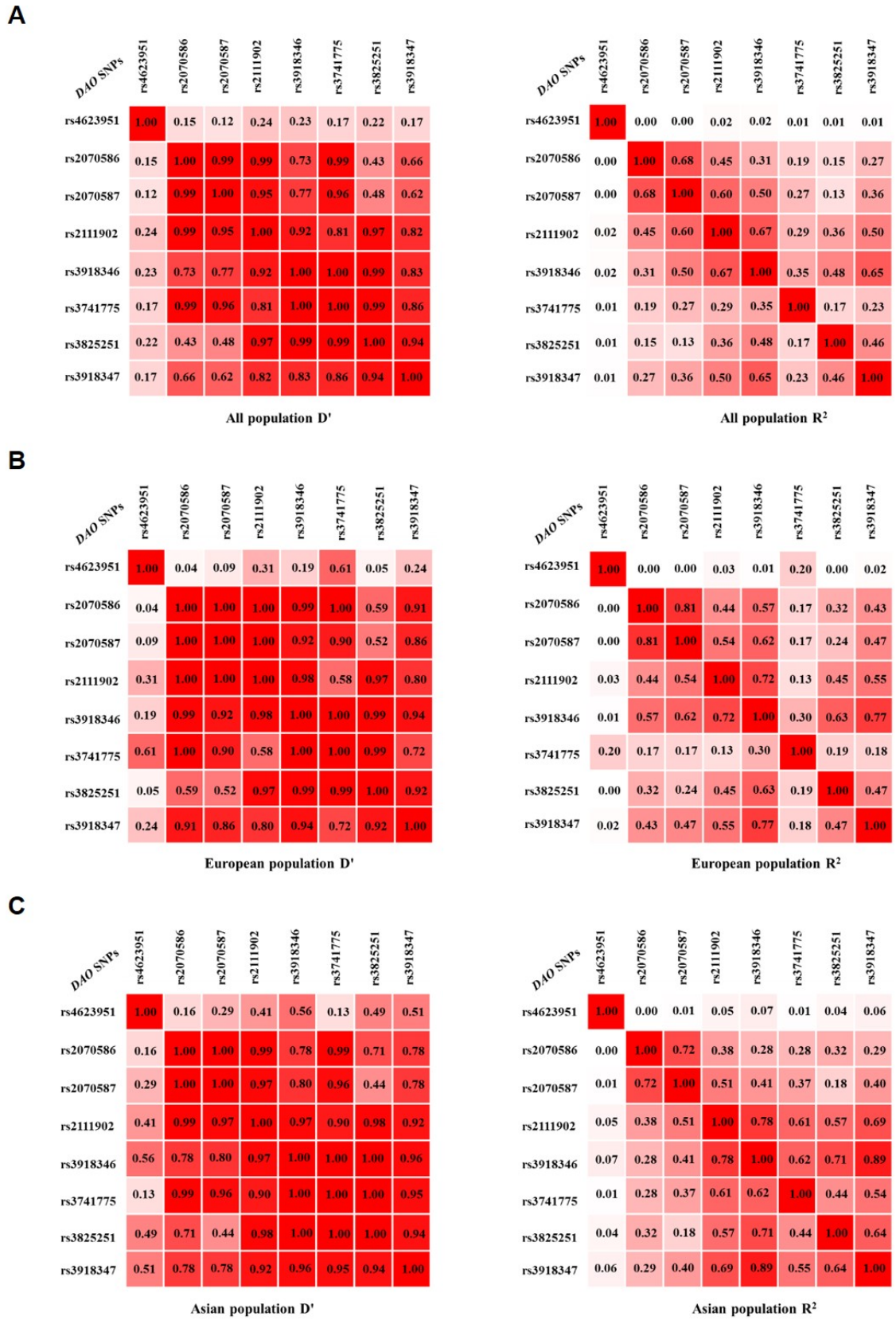


Supplementary Figure S8: Forest plots of the association between *NRG1* polymorphisms and schizophrenia in the allele model. Forest plots of all, only Caucasian and only Asian studies showing association between *NRG1* rs2954041 (**A**), rs6994992 (**B**), SNP8NRG221132 (rs73235619) (**C**), rs776401 (**D**), rs35753505 (**E**), rs6988339 (**F**), SNP8NRG433E1006 (rs113317778) (**G**), rs3924999 (**H**), rs4733376 (**I**), rs7014762 (**J**), rs2439272 (**K**), and rs2466058 (**L**) polymorphisms and schizophrenia; *$p<0.05$ (significant). There was no significant association found between above mentioned *NRG1* polymorphisms and schizophrenia.

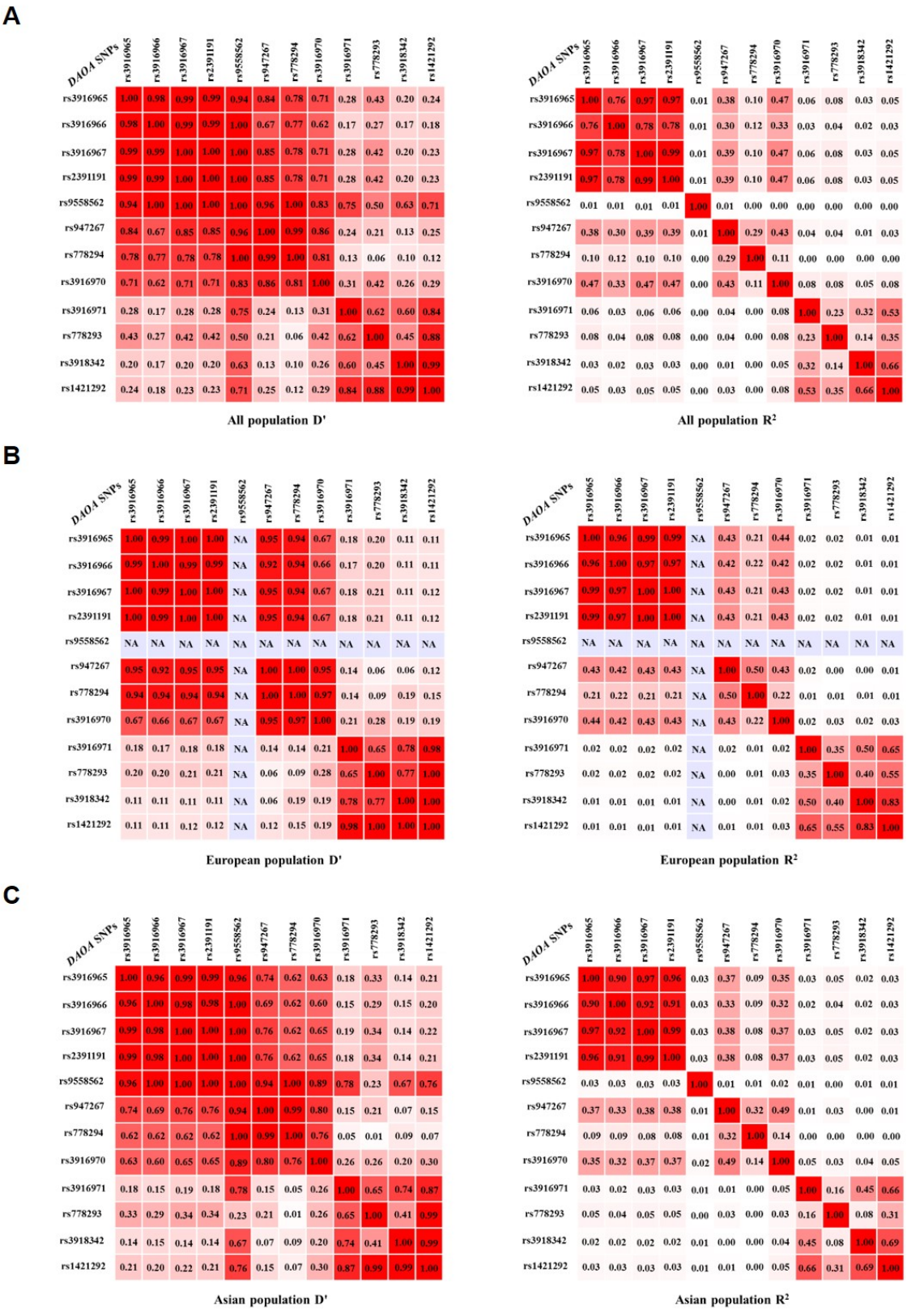

A
NRG1 (SNP8NRG241930): T vs. G
All studies
I²=30%, p=0.09
NRG1 (SNP8NRG241930): T vs. G
Caucasian studies
I²=37%, p=0.08
NRG1 (SNP8NRG241930): T vs. G
Asian studies
I²=23%, p=0.25
B
NRG1 (rs6994992): T vs. C
All studies
I²=49%, p=0.003*
NRG1 (rs6994992): T vs. C
Caucasian studies
I²=55%, p=0.01*
NRG1 (rs6994992): T vs. C
Asian studies
I²=41%, p=0.07
C
NRG1 (rs35753505): C vs. T
All studies
I²=39%, p=0.02*
NRG1 (rs35753505): C vs. T
Caucasian studies
I²=52%, p=0.00
NRG1 (rs35753505): C vs. T
Asian studies
I²=9%, p=0.36
D
NRG1 (rs6988339): G vs. A
All studies
I²=57%, p=0.04*
NRG1 (rs6988339): G vs. A
Caucasian studies
I²=62%, p=0.07
NRG1 (rs6988339): G vs. A
Asian studies
I²=53%, p=0.12
E
NRG1 (SNP8NRG221132): A vs. G
All/Caucasian studies
I²=0%, p=0.49
F
NRG1 (SNP8NRG433E1006): A vs. G
All studies
I²=56%, p=0.046*
NRG1 (SNP8NRG433E1006): A vs. G
Caucasian studies
I²=64%, p=0.02*
Precision
OR



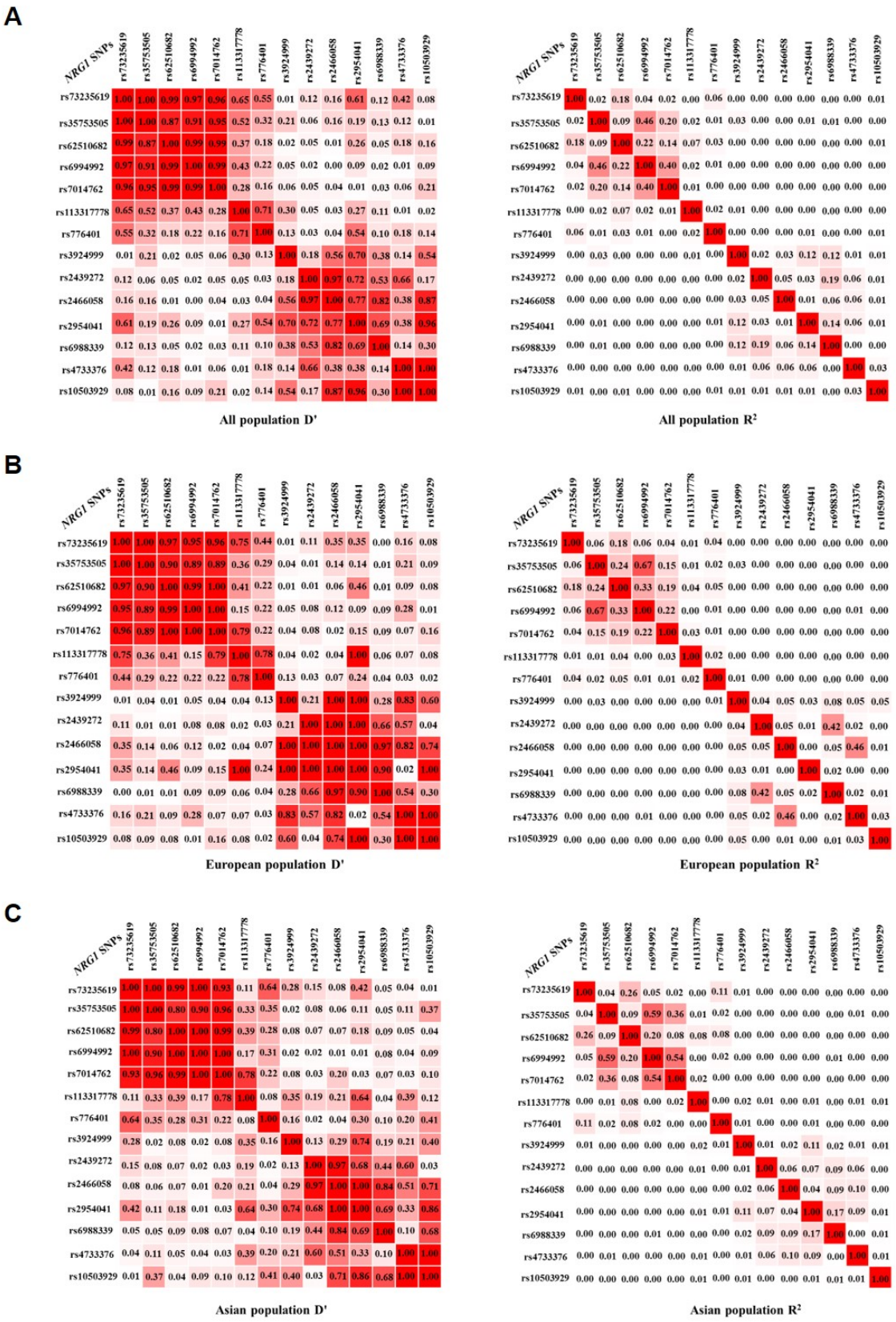
Supplementary Figure S9: Heterogeneity funnel plots of *NRG1* SNP8NRG241930 (rs62510682) (**A**), rs6994992 (**B**), rs35753505 (**C**), rs6988339 (**D**), SNP8NRG221132 (rs73235619) (**E**), SNP8NRG433E1006 (rs113317778) (**F**), rs3924999 (**G**), rs2954041 (**H**), rs776401 (**I**), rs2439272 (**J**), rs2466058 (**K**), rs4733376 (**L**), rs7014762 (**M**), and rs10503929 (**N**) polymorphisms in all studies, only Caucasian and only Asian samples. The heterogeneity between studies was assessed by inconsistency index (I^2), and was considered statistically significant if $p<0.05$ (*).



Supplementary Figure S10: Linkage disequilibrium (LD) plots of *DAO* SNPs from LDlink databases. Linkage disequilibrium plots with D′ and R^2 values across all (**A**), European (**B**), and Asian (**C**) populations. D′ and R^2 values are mentioned inside the squares of the LD plots.



Supplementary Figure S11: Linkage disequilibrium (LD) plots of *DAOA* SNPs from LDlink databases. Linkage disequilibrium plots with D' and R^2 values across all (**A**), European (**B**), and Asian (**C**) populations. D' and R^2 values are mentioned inside the squares of the LD plots; NA: Not calculated.



Supplementary Figure S12: Linkage disequilibrium (LD) plots of *NRG1* SNPs from LDlink databases. Linkage disequilibrium plots with D′ and R^2 values across all (**A**), European (**B**), and Asian (**C**) populations. D′ and R^2 values are mentioned inside the squares of the LD plots.

5.2.7 Acknowledgements

This project was supported by the Swiss Government Excellence Scholarship (2014.0826) to VJ.

5.3 Study III: Prediction analysis for transition to schizophrenia in individuals at clinical high-risk for psychosis: The relationship of *DAO*, *DAOA* and *NRG1* variants with negative symptoms and cognitive deficits

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[8] Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

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Keywords: DAO/DAAO, DAOA/G72, NRG1, APSS, SNP, RDoC

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Author contributions

WR, AT, KH and SW designed the genetic part of the ZInEP study; AT, MG, SW and EG designed the present genetic glutamatergic study. AT, MG, and MF collected the data and blood samples. VJ performed experiments, analysed data, and drafted the manuscript. AT, MG, MF, KH, CUC, WR, SW and EG reviewed the manuscript. All authors contributed to and have approved the final manuscript.

5.3.1 Abstract

Schizophrenia is characterized by positive and negative symptoms and cognitive dysfunction. The glutamate hypothesis of schizophrenia has been hypothesized to explain the negative symptoms and cognitive deficits better than the dopamine hypothesis alone. Therefore, we aimed to evaluate whether glutamatergic variants such as D-amino acid oxidase (*DAO*), DAO activator (*DAOA*)/*G72*, and Neuregulin 1 (*NRG1*) single nucleotide polymorphisms (SNPs) and their mRNA levels predicted (i) transition to schizophrenia-spectrum disorders, and (ii) Research Domain Criteria (RDoC) domains, mainly negative valence and cognitive systems. In a 3-year prospective study cohort of 185 individuals (age: 13-35 years) at high risk (HR) and ultra-high risk (UHR) for psychosis, we assessed *DAO* (rs3918347, rs4623951), *DAOA* (rs778293, rs3916971, rs746187), and *NRG1* (rs10503929) SNPs and their mRNA expression. Furthermore, we investigated their association with RDoC domains, mainly negative valence (e.g., anxiety, hopelessness) and cognitive (e.g., perception disturbances, disorganized symptoms) systems. *NRG1* rs10503929 CC+CT versus TT genotype carriers experienced significantly more disorganized symptoms. *DAOA* rs746187 CC versus CT+TT genotype, *DAOA* rs3916971 TT versus TC+CC genotype, and *DAO* rs3918347 GA+AA versus GG genotype carriers experienced nominally more hopelessness, visual perception disturbances, and auditory perception disturbances, respectively. The schizophrenia risk G-allele of *DAO* rs3918347 nominally increased risk for those UHR individuals with attenuated positive symptoms syndrome. No association between *DAO*, *DAOA*, *NRG1* SNPs and conversion to schizophrenia-spectrum disorders was observed. Our findings suggest that *DAO*, *DAOA* and *NRG1* polymorphisms might influence both RDoC negative valence and cognitive systems, but not transition to schizophrenia-spectrum disorders.

5.3.2 Introduction

Schizophrenia is a chronic and debilitating disorder, preceded by a broad range of symptoms. The emergence of psychotic features in schizophrenia is typically between the late teens and mid-30s (McGrath et al., 2008). Early recognition of individuals at-risk for psychosis and the provision of early intervention is likely to be associated with improved outcomes (Stafford et al., 2013). Individuals at clinical risk for psychosis are identified by two complementary approaches: the high risk (HR) and the ultra-high risk (UHR) criteria. The HR concept is based on basic symptoms and comprises of two partially overlapping risk constellations, the cognitive-perceptive basic symptoms (COPER) and the cognitive disturbances (COGDIS) (Schultze-Lutter et al., 2015, 2012). The UHR criteria comprises of attenuated positive symptoms syndrome (APSS), brief limited intermittent psychotic symptoms (BLIPS), and a combination of a risk factor for psychosis and a recent functional decline (McGlashan et al., 2010). A meta-analysis of 27 studies showed that 18%, 21%, 27% and 32% of individuals at-risk for psychosis (HR+UHR) transitioned to psychotic disorders at 6, 12, 24 and 36 months of follow-up, respectively (Fusar-Poli et al., 2012). This meta-analysis also showed that the mean transition risk was 49%, 28%, and 22% using the HR approach, UHR approach, and when combining both HR and UHR approaches, respectively (Fusar-Poli et al., 2012). One study reported that about one-third of individuals at UHR for psychosis transitioned to psychosis (Gee and Cannon, 2011). Our recent study, using a multivariable prediction model, demonstrated that as expected, UHR criteria predicted conversion to psychosis but combining HR and UHR criteria in this help-seeking at risk population did not improve the predictive accuracy of UHR alone (Hengartner et al., 2017). It is therefore important to optimise the identification of individuals at HR/UHR for psychosis by minimising the false positive rate and improving the true positive prediction rate of conversion to psychosis.

The estimated heritability in schizophrenia is around 60-80% (Pepper and Cardno, 2014). Studies have demonstrated an association between schizophrenia and D-amino acid oxidase (*DAO*), DAO activator (*DAOA*)/*G72*, and Neuregulin 1 (*NRG1*) single nucleotide polymorphisms (SNPs) (Allen et al., 2008; Jagannath et al., 2017a). *DAOA* and *NRG1* polymorphisms were shown to predict the transition to schizophrenia in individuals at HR/UHR for psychosis (Bousman et al., 2013; Keri et al., 2009; Mössner et al., 2010). Genetic studies in schizophrenia have shown association of neurocognitive endophenotypes with several glutamatergic gene polymorphisms including *NRG1* (Greenwood et al., 2011, 2012; Stevenson et al., 2016). These studies suggest that genetic variations

and neurocognitive endophenotypes may help to improve the prediction accuracy of clinical symptoms and HR/UHR criteria for transition in an at-risk population.

The glutamate hypothesis of schizophrenia originated from the observation that N-methyl-D-aspartate (NMDA) receptor blockers like ketamine, induced schizophrenia-like symptoms. As antipsychotics (dopamine D2 receptor antagonists) have little effect on negative symptoms and cognitive deficits, the glutamatergic system is an attractive therapeutic target (Javitt, 1999). Meta-analyses have reported that addition of NMDA receptor agonist D-serine and glycine transporter type 1 inhibitor sarcosine as an adjunct to antipsychotics reduce total and negative symptoms (Singh and Singh, 2011; Tiihonen and Wahlbeck, 2006). Based on these observations, the glutamate hypothesis is thought to describe the pathophysiology underlying negative symptoms and cognitive deficits better than the dopamine hypothesis (Merritt et al., 2013; Veerman et al., 2014; Frohlich and Van Horn, 2014). NMDA receptor hypofunction might lead to decreased dopamine activity in the mesocortical pathway, which may manifest as negative symptoms and cognitive dysfunction in schizophrenia (Stahl et al., 2007). The NMDA receptor hypofunction theory proposed in schizophrenia might be partly explained by increased DAO activity modulated by DAOA leading to decreased D-serine, a co-agonist of NMDA receptors (Sacchi et al., 2016). The function of NRG1 is mediated by binding to receptor tyrosine kinases called ErbB (ErbB3 and ErbB4), and an altered NRG1/ErbB4 signaling is thought to result in NMDA receptor hypofunction (Li et al., 2007; Mei and Nave, 2014). These studies highlight the potential pathogenic link between NMDA receptor hypofunction and dysregulation of *DAO*, *DAOA*, and *NRG1* genes.

The National Institute of Mental Health started the Research Domain Criteria (RDoC) initiative to guide and organize research in psychiatric disorders beyond the typical diagnostic classification approach (Insel et al., 2010). This initiative provides a non-disease-based structured conceptual framework to understand the dimensional range of human behavior from normal to abnormal by integrating multiple levels of information (from genomics to self-reports). The RDoC represents a paradigm shift from Diagnostic and Statistical Manual of Mental Disorders (DSM)/International Classification of Diseases (ICD) to dimensional approaches with an aim to integrate basic research and psychopathology (Cuthbert, 2014).

In this study, we aimed (1) to identify predictive glutamatergic genetic

polymorphisms in at-risk individuals for transition to schizophrenia-spectrum disorders, (2) to identify endophenotypes potentially linked to the glutamatergic system in at-risk individuals using RDoC constructs, and (3) to evaluate differences in *DAO*, *DAOA*, and *NRG1* mRNA levels across clinical and RDoC domains.

5.3.3 Materials and methods

Study population

Participants were recruited by the "Early Recognition and Intervention Program for Psychosis and Bipolar Disorder" project as part of the Zurich Program for Sustainable Development of Mental Health Services (ZInEP; www.zinep.ch) in the Canton of Zurich, Switzerland. The detailed study design, inclusion, and exclusion criteria were described in our previous studies (Metzler et al., 2014; Theodoridou et al., 2014). A total of 185 individuals aged 13-35 years at HR/UHR for psychosis were assessed at baseline and were subsequently followed-up at 6, 12, 24, and 36 months for the transition to schizophrenia. At 36 months follow-up, 50% of individuals (n=93) dropped out of the study. The dropouts were due to refusal to participate in the study in most of the cases and due to non-response after contacting in few cases. However, we are unable to give exact numbers of individuals who refused to participate or who did not respond due to missing information in several study participants. Individuals at HR for psychosis were assessed using the Schizophrenia Proneness Instrument-Child and Youth version (SPI-CY) (age $<$ 18 years) (Schultze-Lutter and Koch, 2009; Fux et al., 2013) or Schizophrenia Proneness Instrument-Adult version (SPI-A) (age $\geq$ 18 years) (Schultze-Lutter et al., 2007), and were included when they had one cognitive-perceptive (COPER) basic symptom or at least two cognitive disturbances (COGDIS). Individuals at UHR for psychosis were assessed using the Structured Interview for Prodromal Syndromes (SIPS) (McGlashan et al., 2001; Miller et al., 2003) and were included when they met criteria for the attenuated positive symptoms syndrome (APSS) or the brief limited intermittent psychotic symptom (BLIPS) or the state-trait criteria (>30% reduction in global assessment of functioning in the past year plus either schizotypal personality disorder or a first degree relative with psychosis). The transition to schizophrenia was defined according to ICD-10 criteria (World Health Organization, 1992). The severity of positive and negative symptoms was assessed using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987), severity of depressive symptoms was assessed with the Calgary Depression Rating scale for Schizophrenia (CDSS) (Addington et al., 1992), and anxiety symptoms with the Beck Anxiety Inventory (BAI) (Beck et al., 1988). The demographic and diagnostic characteristics of the study population are shown in Supplementary Table S1. This study was approved by the Cantonal Ethic Commission of Zurich (Ref. Nr. EK: E-63/2009) and complies with the Declaration of Helsinki. Informed written consent was obtained from adult participants and legal guardians of minors, and written assent was obtained

from minors.

Phenotypic domains

The participants were grouped into clinical phenotypes (cases versus controls) namely, converters to schizophrenia-spectrum disorders (n=27), i.e., schizophrenia, schizophreniform disorder, and acute psychotic disorder, versus non-converters (n=65) at 36 months follow-up [dropouts (n=93)], and APSS (n=98) group versus all other help-seeking individuals (BLIPS, state-trait criteria, COGDIS, COPER) (n=87) at baseline. The transition to schizophrenia was defined according to ICD-10 criteria (World Health Organization, 1992).

First, we grouped our cohort as per the factor structure of the PANSS, concentrating on negative symptoms and general psychopathology (Kay et al., 1987). As we did not find any significant differences in negative symptoms (sum score of negative (N1-N7) PANSS subscale) and general psychopathology (sum score of general psychopathology (G1-G16) PANSS subscale) across *DAO*, *DAOA*, and *NRG1* SNPs, we then decided to use an exploratory approach by focussing on subgroups of psychopathology constructs defined according to the RDoC domains. The RDoC framework consists of five domains namely, negative valence systems, positive valence systems, cognitive systems, systems for social process, and arousal/regulatory systems (Cuthbert and Insel, 2013). In this study, we decided to concentrate on two RDoC domains: negative valence systems and cognitive systems, due to the potential role of *DAO*, *DAOA* and *NRG1* polymorphisms in the glutamate hypothesis of schizophrenia, as it appears to explain the pathogenesis of negative and cognitive symptoms better than the dopamine hypothesis (Hu et al., 2015; Veerman et al., 2014) and unavailability of relevant neuropsychological scales in our study to create the positive valence domain. Negative valence systems focus on responses to aversive situations, such as fear, anxiety and loss. Cognitive systems concentrate on various cognitive processes, such as perception, language, memory, and cognitive control. Within negative valence systems, we focused on the constructs of threat (acute and sustained) and loss. The negative valence system construct of threat was assessed by the total score of BAI with higher BAI scores pointing to increased severity of anxiety (Beck et al., 1988). The negative valence system construct loss was assessed by the response to the CDSS item 2 "hopelessness" which can be scored as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) (Addington et al., 1992). Within cognitive systems, we chose the constructs of perception (visual and auditory) and cognitive control. The cognitive system construct visual perception was assessed by summing the following four SPI-A (and equivalent SPI-CY) items O4 (other visual perception disturbances), F1

(hypersensitivity to light), F2 (photopsia), and F3 (micropsia/macropsia), with higher scores pointing to more frequent disturbances in visual perception. The cognitive system construct of auditory perception was assessed by summing the following three SPI-A (and equivalent SPI-CY) items O5 (other acoustic perception disturbances), F4 (hypersensitivity to sounds/noise), and F5 (changed intensity/quality of acoustic stimuli), with higher scores pointing to more frequent disturbances in auditory perception. The cognitive system construct of cognitive control was obtained by summing the following four SIPS disorganization items D1, D2, D3, and D4 (McGlashan et al., 2001; Miller et al., 2003), with higher scores indicating more severe disturbance in disorganised symptoms.

The above-mentioned scales used to tap into RDoC domains were assessed at baseline and last-available follow-up until 36 months. As there was a dropout rate of 50% at 36 months, if there were no data available at 36 months, we took the scores from the last follow-up that the individual attended (i.e., 6 or 12 or 24 months).

TaqMan single nucleotide polymorphism (SNP) genotyping

The study population was genotyped for *DAO* (rs3918347, rs4623951), *DAOA* (rs778293, rs3916971, rs746187), and *NRG1* (rs10503929) SNPs. These SNPs were selected based on previously reported significant association with schizophrenia (Allen et al., 2008; Mechelli et al., 2012). In our recent meta-analysis, we found a significant association of *DAO* rs4623951 [odds ratio (OR)=0.88; minor allele: C), *DAOA* rs778293 (OR=1.17; minor allele: G), *DAOA* rs3916971 (OR=0.84; minor allele: T), and *NRG1* rs10503929 (OR=0.89; minor allele: C) with schizophrenia (Jagannath et al., 2017a). In this study, the carriers of the risk allele of *DAO*, *DAOA*, and *NRG1* SNPs were anticipated to have worse psychopathology scores in RDoC negative valence and cognitive system constructs. DNA was isolated from whole blood Ethylenediaminetetraacetic acid (EDTA) tubes collected from the study population using QIAamp DNA Blood Maxi Kit (Qiagen) as per manufacturer's protocol. A spectrophotometer (NanoVue Plus, GE) was used to measure DNA concentrations, A260/A280, and A260/A230 ratios. The study population was genotyped for *DAO* (rs3918347 assay number: C_27937201_10, rs4623951 assay number: C_32177440_10, both from Applied Biosystems, USA), *DAOA* (rs778293 assay number: C_8704507_10, rs3916971 assay number: C_27495752_10, rs746187 assay number: C_1925241_10, all from Applied Biosystems, USA), and *NRG1* (rs10503929, assay number: C_2870393_10, Applied Biosystems, USA) SNPs (Jagannath et al., 2017b). DNA (10 ng/μl), TaqMan® Genotyping Master Mix (Applied Biosystems, USA),

and above-mentioned *DAO*, *DAOA*, *NRG1* SNP Genotyping Assays (Applied Biosystems, USA) were combined in a 384-well plate. Real-time PCR was performed in a C1000^{TM}CFX384^{TM} Thermal cycler (Bio-Rad) using TaqMan® SNP Genotyping Assay PCR standard protocol. The allelic discrimination program of Bio-Rad CFX Manager^{TM} Software version 2.1 was used to determine genotypes (Jagannath et al., 2017b). Samples were run in duplicates to ensure correct results. In case of ambiguity in duplicates, genotyping was repeated in a separate run to ensure correct results. No-template controls (NTC) were included in every run to exclude impurities.

Quantification of *NRG1*, *DAO*, and *DAOA* mRNA levels using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was isolated from whole blood collected from the study population using PAXgene Blood RNA Kit (Qiagen) according to manufacturer's protocol. A spectrophotometer (NanoVue Plus, GE) was used to measure RNA concentrations, A260/A280, and A260/A230 ratios. RNA integrity was analysed using Experion automated electrophoresis system (Bio-Rad) in a subset of samples to ensure RNA Integrity Number (RIN)/RNA quality Indicator (RQI) $>$ 7. RNA (500 ng) was reverse transcribed using iScript^{TM} cDNA Synthesis Kit (Bio-Rad) as per manufacturer's protocol. In a subset of samples, negative controls were prepared with RNA using iScript^{TM} cDNA Synthesis Kit (Bio-Rad) without reverse transcriptase enzyme as per manufacturer's protocol. qRT-PCR was performed using cDNA, QuantiFast SYBR Green PCR kit (Qiagen), 1 μM *NRG1* primer (QT00061964, Qiagen), and reference genes (β-actin (*ACTB*) (QT01680476), aminolevulinate synthetase (*ALAS1*) (QT00073122), ribosomal protein L13a (*RPL13A*) (QT00089915), alanyl-tRNA synthetase (*AARS*) (QT00054747), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (QT01192646), peptidyl prolyl isomerase A (*PPIA*) (QT00866137), and X-prolyl aminopeptidase1 (*XPNPEP1*) (QT00051471); all from Qiagen). *NRG1* mRNA levels were normalised to the references genes (Jagannath et al., 2017b). PCR efficiencies were calculated using LinReg PCR program (Ruijter et al., 2009) and mean PCR efficiencies for all studied amplicons were found to be between 91% and 93%. Normalised *NRG1* mRNA levels were quantified using qBASE plus software (Biogazelle) which utilises gene-specific amplification efficiencies, and allows normalisation with multiple reference genes (Hellemans et al., 2007).

We performed qRT-PCR to detect *DAO* mRNA using *DAO* primers described by Verrall et al.,(Verrall et al., 2007) and pre-designed primers (qHsaCID0011122 and qHsaCEP0058247 (Bio-Rad), Hs.PT.58.3248433 and Hs.PT.58.45768871 (IDT)). We performed qRT-PCR to detect *DAOA* mRNA using primers for *DAOA*

gene described by Benzel et al., (Benzel et al., 2008), Cheng et al., (Cheng et al., 2014), and pre-designed primers (QT00058863 (Qiagen), Hs.PT.58.555086 (IDT), 4331182 (ThermoFisher scientific), qHsaCEP0024792 (Bio-Rad)). QuantiTect Whole Transcriptome Kit (207043, Qiagen) followed by qRT-PCR was also used to detect *DAO* and *DAOA* mRNA levels (Jagannath et al., 2017b). However, we were unable to quantify *DAO* and *DAOA* mRNA with the aforementioned methods in the whole blood as either no signal was observed or genomic DNA was amplified concomitantly.

Statistical analysis

The results from SNP genotyping was analysed using PLINK software (Purcell et al., 2007). The minor allele frequency and deviation from Hardy-Weinberg Equilibrium (HWE) was computed using PLINK software, and $p<0.05$ was considered as statistically significant. The *DAO* (rs3918347), *DAOA* (rs3916971, rs778293, rs746187), and *NRG1* (rs10503929) SNPs were in Hardy-Weinberg equilibrium ($p>0.05$), and the minor allele frequency (MAF) of *DAO*, *DAOA*, and *NRG1* SNPs were similar to HapMap CEU MAF (Supplementary Table S5). The *DAO* rs4623951 SNP deviated from the Hardy-Weinberg equilibrium ($p<0.05$) (Supplementary Table S4). The differences in allele and genotype frequencies across clinical phenotypes (cases versus controls) were assessed using Chi-square test, $p<0.05$ was taken as statistically significant. The OR, 95% CI, and p-value for SNP models (allelic, dominant and recessive) across clinical phenotypes were calculated from allele/genotype frequencies using an online OR calculator (https://www.medcalc.org/calc/odds_ratio.php), and p-value was adjusted based on the number of analysed SNPs (Bonferroni correction, $p<0.008$). The post hoc power analyses for association of *DAO*, *DAOA*, and *NRG1* SNPs with converters to schizophrenia-spectrum disorders versus non-converters (Supplementary Table S5) and APSS versus all other help-seeking group were conducted using Fisher's exact test of independence in G*Power software (Faul et al., 2009), the calculated OR was used, and the alpha level was set at 0.05.

IBM® SPSS® Statistics (version 21) software was used for statistical analysis. Shapiro-Wilk test with Lilliefors significance correction was used to assess the normality of the distribution of *NRG1* gene expression and clinical scales (BAI, CDSS, SPI-A/SPI-CY, SIPS, PANSS). *NRG1* gene expression and clinical scales showed both normal and non-normal distribution. We used non-parametric tests even for normally distributed data in order to maintain consistency between statistical evaluations. The differences in RDoC domains (negative valence and cognitive systems) across models (genotypic, dominant, recessive) were assessed using Mann-Whitney test (for 2 groups) or Kruskal-Wallis test (for >2

groups), p-values were adjusted based on the number of constructs analysed (Bonferroni correction, $p<0.008$). The differences in *NRG1* mRNA levels across clinical phenotypes were assessed using Mann-Whitney test and $p<0.05$ was set as statistically significant. The post hoc power analyses for RDoC negative valence and cognitive systems across *DAO*, *DAOA*, and *NRG1* SNPs were conducted using analysis of variance (ANOVA) test for 3 groups or t-test for 2 groups in G*Power software (Faul et al., 2009), the effect sizes were determined from means of neuropsychological scales used in RDoC negative valence and cognitive systems, and the alpha level was set at 0.05 (Supplementary Table S7). The differences in *NRG1* mRNA levels across *NRG1* rs10503929 SNP genotypes was assessed using Kruskal-Wallis test and across dominant (CC+CT, TT) and recessive models (CC, CT+TT) were assessed using Mann-Whitney test, with $p<0.05$ being set as statistically significant. The correlation between *NRG1* gene expression and RDoC domains (negative valence and cognitive systems) was assessed using Spearman's rank correlation test and $p<0.05$ was considered statistically significant.

5.3.4 Results

The *DAO* (rs3918347), *DAOA* (rs3916971, rs778293, rs746187), and *NRG1* (rs10503929) SNPs were in Hardy-Weinberg equilibrium ($p>0.05$), and the minor allele frequency (MAF) of *DAO*, *DAOA*, and *NRG1* SNPs were similar to HapMap CEU MAF (Supplementary Table S2).

DAO, *DAOA*, and *NRG1* polymorphisms across clinical phenotypes

There were no significant associations between *DAO*, *DAOA*, and *NRG1* SNPs with converters to schizophrenia-spectrum disorders compared to non-converters at 36 months follow-up (power range: 0.05-0.31; Supplementary Table S3). However, there was a nominal association ($p>0.008$) of *DAO* rs3918347 with APSS compared to all other help-seeking group at baseline, and the G-allele had a tendency to be a risk allele for APSS (OR=1.84, 95% CI=1.13-3.01, p=0.01), with a power of 0.76 (Supplementary Table S4). There were no significant association between the of rest of the *DAO*, *DAOA*, *NRG1* SNPs and APSS (power range: 0.05-0.89; Supplementary Table S4).

DAO, *DAOA*, and *NRG1* polymorphisms across RDoC domains

DAOA rs746187 recessive (CC) genotype carriers experienced nominally more hopelessness (higher item 2 score, CDSS) than CT+TT genotype carriers at the last available follow-up time point (LA) until 36 months (p=0.04) analysed by Mann-Whitney test (RDoC negative valence system: loss; Figure 1; power=0.62; effect size=0.55; Supplementary Table S8). *DAOA* rs3916971 recessive (TT) genotype carriers experienced nominally more disturbances in visual perception (higher sum of O4+F1+F2+F3, SPI-A) than TC+CC genotype carriers at LA until 36 months (p=0.009) analysed by Mann-Whitney test (RDoC cognitive system: visual perception; Figure 2; power=0.28; effect size=0.34; Supplementary Table S8). Individuals with *DAOA* rs3916971 TC+CC versus TT genotype improved and had fewer visual perceptual disturbances at LA until 36 months compared to baseline, but at LA until 36 months versus baseline, they continued to have less visual perceptual disturbances than individuals with TT genotype (Figure 2C). There were no significant differences in negative valence (threat; Supplementary Table S5; power range: 0.05-0.72, Supplementary Table S8), auditory perception disturbances (Supplementary Table S6; power range: 0.05-0.56, Supplementary Table S8), and cognitive control (Supplementary Table S7; power range: 0.05-0.31, Supplementary Table S8) in individuals with *DAOA* SNP genotypes (rs3916971, rs778293, rs746187).

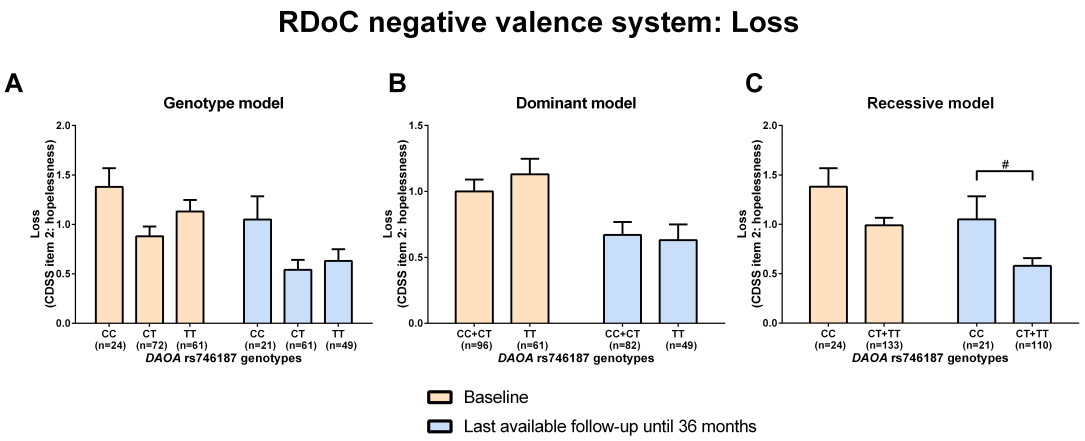

Figure 1: Differences in RDoC negative valence system loss across *DAOA* rs746187 genotypes. Differences in Calgary Depression Scale for Schizophrenia (CDSS) item 2: hopelessness scale across *DAOA* rs746187 genotypes in genotype (**A**), dominant (**B**), and recessive (**C**) model at baseline and last available follow-up time point until 36 months. Data is presented as mean±SEM; *p<0.008 (significant with Bonferroni correction); #0.008<p<0.05 (significant without Bonferroni correction).

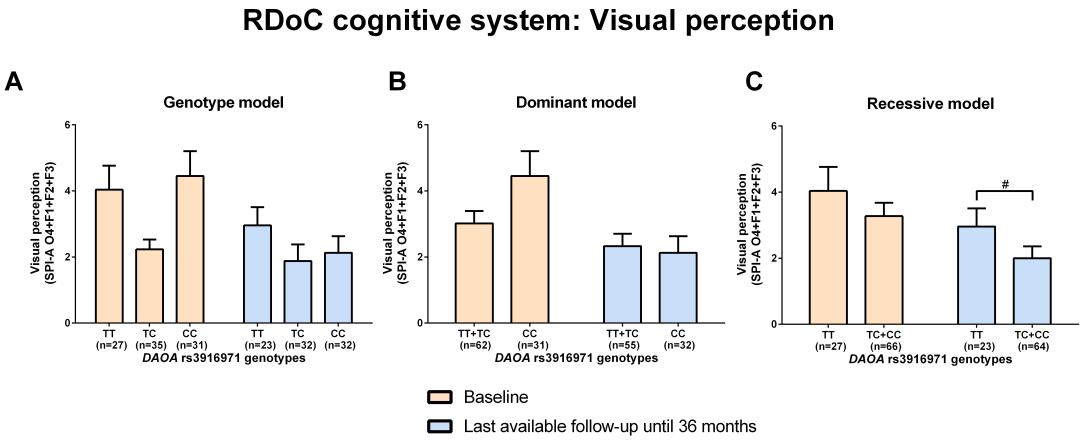

Figure 2: Differences in RDoC cognitive system visual perception across *DAOA* rs3916971 genotypes. Differences in Schizophrenia Proneness Instrument-Adult version (SPI-A) O4+F1+F2+F3 sum score across *DAOA* rs3916971 genotypes in genotype (**A**), dominant (**B**), and recessive (**C**) model at baseline and last available follow-up time point until 36 months. Data is presented as mean±SEM; *p<0.008 (significant with Bonferroni correction); #0.008<p<0.05 (significant without Bonferroni correction).

DAO rs3918347 GA+AA genotype carriers experienced nominally (p=0.04) more disturbances in auditory perception (higher sum of O5+F4+F5, SPI-A) than GG genotype carriers at baseline analysed by Mann-Whitney test (RDoC cognitive system: auditory perception; Figure 3; power=0.41; effect size=0.44; Supplementary Table S8). Individuals with *DAO* rs3918347 GA+AA versus GG genotype improved and had lesser auditory perceptual disturbances at LA until 36 months compared to baseline, but at LA until 36 months versus baseline, they

continued to experience more auditory perceptual disturbances than individuals with CC genotype (Figure 3C). We did not find significant differences in negative valence systems (anxiousness and hopelessness; Supplementary Table S5; power range: 0.05-0.92, Supplementary Table S8), visual perception disturbances (Supplementary Table S6; power range: 0.05-0.57, Supplementary Table S8), and cognitive control (Supplementary Table S7; power range: 0.05-0.22, Supplementary Table S8) in individuals with *DAO* (rs3918347, rs4623951) SNP genotypes.

NRG1 rs10503929 CC+CT genotype carriers had significantly (p=0.001) more disorganised symptoms (higher sum of the D1-D4 score, SIPS) than TT genotype carriers at baseline analysed by Mann-Whitney test (RDoC cognitive system: cognitive control; Figure 4; power=0.99; effect size=0.59; Supplementary Table S8). There were no significant differences in negative valence systems (anxiousness and hopelessness; Supplementary Table S5; power range: 0.07-0.51, Supplementary Table S8), and visual and auditory perception disturbances (Supplementary Table S6; power range: 0.05-0.36, Supplementary Table S8) in individuals with *NRG1* (rs10503929) SNP genotypes.

To analyse the effect of age on RDoC domains across *DAO*, *DAOA*, and *NRG1* SNPs, we performed an analysis of covariance (ANCOVA) with age as a covariate after transformation of not normally distributed BAI, SPI-A, and SIPS data, as described previously (Templeton, 2011). We did not find a significant effect of age on differences in RDoC domains across *DAO*, *DAOA*, and *NRG1* SNPs (data not shown).

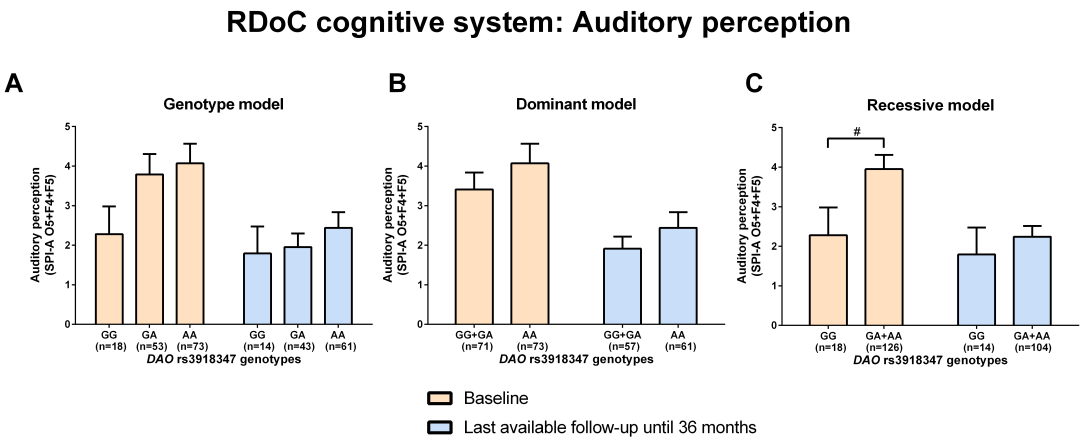

Figure 3: Differences in RDoC cognitive system auditory perception across *DAO* rs3918347 genotypes. Differences in Schizophrenia Proneness Instrument-Adult version (SPI-A) O5+F4+F5 sum score across *DAO* rs3918347 genotypes in genotype (**A**), dominant (**B**), and recessive (**C**) model at baseline and last available follow-up time point until 36 months. Data is presented as mean±SEM; *$p<0.008$ (significant with Bonferroni correction); $^{\#}0.008<p<0.05$ (significant without Bonferroni correction).

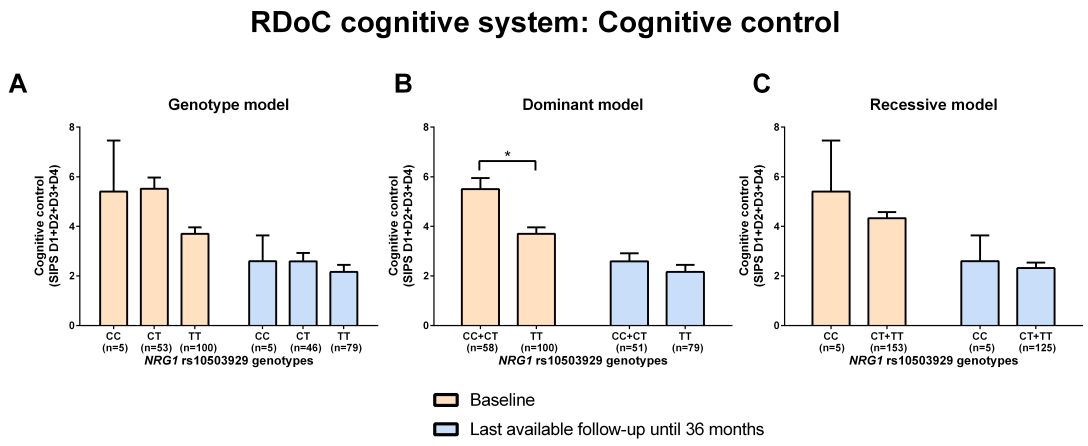

Figure 4: Differences in RDoC cognitive system cognitive control across *NRG1* rs10503929 genotypes. Differences in Structured Interview for Prodromal Syndromes (SIPS) sum score of D1-D4 across *NRG1* rs10503929 genotypes in genotype (**A**), dominant (**B**), and recessive (**C**) model at baseline and last available follow-up time point until 36 months. Data is presented as mean±SEM; *$p<0.008$ (significant with Bonferroni correction); $^{\#}0.008<p<0.05$ (significant without Bonferroni correction).

NRG1, *DAO* and *DAOA* mRNA expression across RDoC and clinical domains

There was a significant positive correlation between increased *NRG1* mRNA levels and higher scores on the RDoC negative valence system loss at LA until 36 months (Supplementary Table S9). Apart from this, there were no significant correlations between *NRG1* mRNA levels and RDoC domains at baseline and at LA until 36 months (Supplementary Table S9). There were no statistically significant differences in *NRG1* mRNA levels across clinical phenotypes (Supplementary Table S10). We did not find significant differences in *NRG1* mRNA levels across *NRG1* rs10503929 SNP genotypes, dominant, and recessive models analysed by Mann-Whitney and Kruskal-Wallis test (Supplementary Table S11). We were unable to quantify *DAO* and *DAOA* mRNA levels in the whole blood of at-risk population.

5.3.5 Discussion

In this 3-year follow-up study of 185 at-risk individuals, *NRG1* rs10503929 CC+CT versus TT genotype carriers experienced significantly more disorganized symptoms, *DAOA* rs746187 CC versus CT+TT genotype, *DAOA* rs3916971 TT versus TC+CC genotype, and *DAO* rs3918347 GA+AA versus GG genotype carriers experienced nominally more hopelessness, visual perception disturbances, and auditory perception disturbances, respectively. Moreover, we found no significant association between *DAO*, *DAOA*, *NRG1* SNPs and conversion to schizophrenia-spectrum disorders, however, we did find a nominally increased risk for APSS with the G-allele of *DAO* rs3918347 carriers.

The *DAO*, *DAOA*, and *NRG1* SNPs did not predict conversion to schizophrenia-spectrum disorders at 36 months follow-up. This lack of association may be due to a low conversion rate of 14.6%, a high drop-out rate of 50% at 36 months follow-up, and that the comparison group of non-converters was not a healthy control group but rather a heterogeneous group of HR and UHR individuals. In this heterogeneous at-risk population, individuals are likely to be on different developmental trajectories of/towards various neuropsychiatric disorders, which might have further complicated the genetic prediction of transition to schizophrenia in our study. Furthermore, our conversion group not only contained patients with schizophrenia, but also patients with other schizophrenia-spectrum disorders. Thus, recruiting a more homogeneous at-risk population would have been more appropriate (Cornblatt and Carrión, 2016; Cornblatt et al., 2015). In contrast to previous meta-analyses showing conversion rates of 29-32% at 36 months follow-up (Fusar-Poli et al., 2012; Schultze-Lutter et al., 2015), our study had a low conversion rate of 14.6%. A study of 82 UHR individuals showed that 100% of the *DAOA* rs1341402 CC genotype carriers (n=4) compared to 50% of the *DAOA* rs778294 AA genotype carriers (n=10; A-allele protective against schizophrenia) progressed to psychosis within 24 months (Mössner et al., 2010). However, a recent study with 225 UHR individuals did not replicate these findings (Bousman et al., 2013). Furthermore, another study of 67 UHR individuals showed that 100% of TT genotype carriers (n=25) of *NRG1* rs62510682 developed psychosis within 12-months (Keri et al., 2009), but this finding was not replicated in the aforementioned study with 225 UHR individuals (Bousman et al., 2013). About 46% of the *NRG1* rs4281084 AA genotype UHR carriers (n=13), and 44% of the T-allele UHR carriers (n=45) transitioned to psychosis within a 15 year follow-up period (Bousman et al., 2013). Thus, there is ambiguity regarding the association of *DAOA* and *NRG1* polymorphisms with the transition to psychosis. We did not assess the

aforementioned *DAOA* and *NRG1* SNPs in this study as we only focussed on the SNPs associated with schizophrenia (Allen et al., 2008; Mechelli et al., 2012).

Our finding that *NRG1* rs10503929 TT genotype carriers had significantly less disorganised symptoms than the dominant (CC+CT) genotype carriers at baseline (RDoC cognitive control) is consistent with previous finding of a significant association of *NRG1* rs10503929 with cognitive domains (abstraction and mental flexibility, attention and verbal memory) in schizophrenia patients, in which the C-allele (protective against schizophrenia) was associated with decreased cognitive performance (Yokley et al., 2012). We found that *NRG1* rs10503929 (CT+TT) versus CC genotype carriers had nominally more auditory perception disturbances. To our knowledge, there are no previous studies on associations between the *NRG1* rs10503929 (exon 8/9/10) and perceptual disturbances. However, a study in adolescents demonstrated that *NRG1* rs3924999 (exon 2) was associated with perceptual disturbances (Lin et al., 2005).

The nominal associations between *DAOA* rs746187 and the RDoC negative valence system: loss, and that between *DAOA* rs3916971 and the RDoC cognitive system: visual perception, points to the possible role of *DAOA* variations in modulating endophenotypes underlying psychosis risk. A recent study found a nominal association of *DAOA* rs3916971 with a psychotic disorder (Andreou et al., 2015). Another study conducted in healthy male controls found that *DAOA* rs3916971 schizophrenia risk C-allele carriers had worse visual-spatial skills (Leach et al., 2013; Crespi and Leach, 2016). In our study, *DAO* rs3918347 GA+AA genotype carriers experienced nominally more auditory perception disturbances than GG genotype carriers at baseline (RDoC cognitive system). Another study found a negative association of *DAO* rs3918346 with neurocognitive functioning in schizophrenia patients (Goldberg et al., 2006). Therefore, the association of *DAO* and *DAOA* SNPs with hopelessness and perception disturbances of our study needs further confirmation.

We further found that *NRG1* mRNA levels increased with higher CDSS hopelessness scores. Previous post-mortem studies have shown increased *NRG1* mRNA levels in the hippocampus (Law et al., 2006) and prefrontal cortex (Chong et al., 2008; Hashimoto et al., 2004) of schizophrenia patients compared to healthy controls. Moreover, studies have shown dysfunctions in these regions might lead to hopelessness (Johnston et al., 2015; Mayberg et al., 1999; Warden et al., 2012).

We examined associations of *DAO*, *DAOA*, and *NRG1* SNPs with risk profiles in individuals at-risk for psychosis. For the sake of higher homogeneity, we also

focussed on the APSS subsample, a classification which the DSM-5 working group included under "conditions for further study" (American Psychiatric Association, 2013). We found that the schizophrenia risk G-allele of *DAO* rs3918347 had a tendency to be a risk allele for APSS compared to the remaining help-seeking group. This result has to be interpreted cautiously because the help-seeking group is not a healthy control group, but a mixed group of BLIPS, state-trait risk criterion, and HR.

We did not find any significant differences in *NRG1* mRNA levels between converters to schizophrenia-spectrum disorders versus non-converters, and APSS versus all other help-seeking non-converters, which might be due to small subsample size leading to modest power (supplementary tables S3 and S4) and heterogeneous subgroups. Our results are in contrast to a study, which showed that *NRG1* (type I and II isoforms) mRNA expression was significantly lower in blood of UHR individuals who transitioned to psychosis (n=31) compared to non-converters (n=66) and controls (n=50) (Kiss et al., 2012). The discrepancy in the results of our study and the aforementioned study might also be due to the differences in isolation method, number of reference genes, stability of the reference genes, and normalisation method used to normalise *NRG1* mRNA levels to reference genes. A study conducted on immortalised lymphocytes showed that there was no difference in *NRG1* mRNA levels between schizophrenia patients and healthy controls (Yamamori et al., 2011).

We were unable to detect *DAO* and *DAOA* mRNA using qRT-PCR in the whole blood of individuals at-risk for psychosis, which is in line with a study which used RNA sequencing to detect *DAO* and *DAOA* mRNA in healthy participants (GTEx Consortium, 2015). As qRT-PCR can detect low copy number genes (Morrison et al., 1998), undetectable *DAO* and *DAOA* mRNA levels might suggest either very low expression below the detection limit of qRT-PCR or extremely localised expression (Jagannath et al., 2017b). The reasons for very low or no expression of *DAO* and *DAOA* mRNA in blood might be highly methylated (75-90%) Illumina CpG sites of *DAO* and *DAOA* genes in healthy individuals (Hannon et al., 2015). Another reason for this low or no expression might be the expression of these genes specifically in the brain (Korostishevsky et al., 2006; Verrall et al., 2007) because of their role in glutamatergic neurotransmission via NMDA receptors (Sacchi et al., 2016).

In our study, *DAO* rs4623951 genotype data showed that the study population deviated from the Hardy-Weinberg Equilibrium (HWE). Since we controlled for genotyping errors, this deviation from HWE might be due to the observed

excess of CT heterozygotes (54%). This excess heterozygosity might be caused by "selection favouring heterozygotes, outbreeding, and negative assortative mating" (Ziegler et al., 2010). As deviation from the HWE creates bias in the associations reported (Salanti et al., 2005), the association results of *DAO* rs4623951 SNP should be interpreted with caution. As this study did not have all the instruments suggested by RDoC for negative valence and cognitive systems, we used an exploratory approach, using the instruments available in this study to create the respective constructs. Thus, future studies are needed to confirm our RDoC findings.

Our study has several limitations, which must be acknowledged. Although a total of 185 individuals at-risk for psychosis were recruited, sample sizes within genotypes across clinical and RDoC domains were small and the power of the study was modest. In this study, only a small percentage (14.6%) of at-risk individuals converted to schizophrenia at 36 months follow-up, 50% of individuals dropped out of the study before 36 months, and there was no healthy control group. The conversion status of the dropouts is unknown, and thus it is not possible to reliably determine the conversion rate. The group of all other help seeking individuals was at heterogeneous risk (BLIPS, state-trait criteria, COPER, COGDIS). The sample size at 36-months follow-up for different psychopathology scales was small because of the high dropout rate. To circumvent this problem, we used the last psychopathology assessment available from each individual. Therefore, the results of LA until 36 months should be interpreted with caution, as they are not based on a homogeneous 36-month follow-up score. We studied only few genes (3 genes) and a relatively small number of their polymorphisms (6 SNPs) which might be the reason for not finding markers for predicting conversion to schizophrenia spectrum disorders. However, these genes and their polymorphisms are still of interest due to their importance in glutamatergic neurotransmission. This study also has strengths, which needs to be highlighted. We recruited individuals from a broad age range (13-35 years) and used age-specific scales (e.g., SPI-A/SPI-CY). Most of the published literature in at-risk population has focussed on clinical phenotypes. The present study used both clinical phenotypes and RDoC domains, especially negative valence and cognitive systems, due to the role of the studied genes in the glutamate hypothesis of schizophrenia.

In summary, although *DAO*, *DAOA*, and *NRG1* SNPs did not emerge as predictive markers for conversion to schizophrenia-spectrum disorders, future association studies with larger cohorts and longer follow-ups are needed to confirm the role of these genes in transition to schizophrenia-spectrum disorders

in the at-risk population. We also identified an association between the studied glutamatergic variants and RDoC negative valence and cognitive systems, which indirectly implicates the role of these genetic variants in the glutamate hypothesis of schizophrenia. Future studies using RDoC domains might help to determine specific endophenotypes within at-risk populations. This might provide clinically useful, genetically informed risk prediction for dimensional and categorical outcomes among populations who maybe at-risk for developing psychosis.

5.3.6 Supplementary Information

Supplementary tables

Supplementary Table S1: Demographics and diagnostic characteristics of the study population

Categorical variables		**Baseline (n=185)**	
		N	**%**
Female		75	40.5
Male		110	59.5
Converters		27	14.6
Non-converters		65	35.1
APSS		98	53
All other help-seeking (n=87)	COPER	28	15.1
	COGDIS	7	3.8
	HR (COPER+COGDIS)	43	23.2
	BLIPS	4	2.2
	State-trait criteria	5	2.7

Continuous variables	**Baseline (n=185)**			**Last available follow-up data until 36 months (n=151)**		
	N	**Range**	**Mean±SEM**	**N**	**Range**	**Mean±SEM**
Age in years	185	13-35	20.51±0.42	151	13-35	20.04±0.23
Threat (sum of BAI scale)	169	0-57	19.6±0.88	139	0-84	12.29±1.07
CDSS: item 2	185	0-3	1.06±0.07	151	0-3	0.67±0.07
Visual perception (SPI-A O4+F1+F2+F3)	111	1-15	3.39±0.32	98	1-15	2.29±0.27
Auditory perception (SPI-A O5+F4+F5)	173	0-16	3.77±0.29	135	0-15	2.17±0.23
Cognitive control (SIPS: sum of D1-D4)	185	0-15	4.46±0.22	150	0-10	2.40±0.20

APSS: attenuated positive symptoms syndrome; COPER: cognitive-perceptive basic symptoms; COGDIS: cognitive disturbances; BLIPS: brief limited intermittent psychotic symptoms; HR: high risk criteria; BAI: Beck Anxiety Inventory; CDSS: Calgary Depression Rating scale for Schizophrenia; SPI-A: Schizophrenia Proneness Instrument-Adult version ($\geq$ 18 years); SPI-CY: Schizophrenia Proneness Instrument-Child and Youth version ($<$ 18 years); SIPS: Structured Interview for Prodromal Symptoms; SEM: standard error of the mean

Supplementary Table S2: Hardy-Weinberg equilibrium and minor allele frequencies of *DAO*, *DAOA* and *NRG1* SNPs in the study population

Gene	SNP ID	Genotype	N	H-W p-value	Minor allele	MAF	HapMap/ NCBI CEU MAF
D-amino acid oxidase (*DAO*)	rs3918347	GG	24	0.09	G	0.3189	0.274
		GA	70				
		AA	91				
	rs4623951	CC	18	**0.04***	C	0.3676	0.412
		CT	100				
		TT	67				
D-amino acid oxidase activator (*DAOA*)	rs3916971	TT	42	0.14	T	0.4462	0.394
		TC	82				
		CC	62				
	rs778293	GG	43	0.18	G	0.4541	0.389
		GA	82				
		AA	60				
	rs746187	CC	26	0.87	C	0.3703	0.375
		CT	85				
		TT	74				
Neuregulin 1 (*NRG1*)	rs10503929	CC	7	0.82	C	0.207	0.225
		CT	63				
		TT	116				

H-W: Hardy-Weinberg; MAF: minor allele frequency; SNP: single nucleotide polymorphism; *p<0.05 (**bold font**)

Supplementary Table S3: Association of *DAO*, *DAOA* and *NRG1* SNPs with converters to schizophrenia-spectrum disorders (n=27) versus non-converters (n=65) at 36 months follow-up and power analysis

Gene	SNP ID	Minor allele	Model	Genotype/ allele	Phenotype		OR	95% CI	p-value	Power[b]
					Converters (N)	Non-converters (N)				
D-amino acid oxidase (*DAO*)	rs3918347	G	Genotypic	GG	2	6	-	-	0.27[a]	-
				GA	10	18				
				AA	8	35				
			Allelic	G	14	30	1.58	0.73-3.41	0.25	0.29
				A	26	88				
			Dominant	GG+GA	12	24	2.19	0.78-6.15	0.14	0.31
				AA	8	35				
			Recessive	GG	2	6	0.98	0.18-5.30	0.98	0.05
				GA+AA	18	53				
	rs4623951	C	Genotypic	CC	3	7	-	-	0.62[a]	-
				CT	9	34				
				TT	8	18				
			Allelic	C	15	48	0.88	0.41-1.83	0.72	0.06
				T	25	70				
			Dominant	CC+CT	12	41	0.66	0.23-1.89	0.44	0.13
				TT	8	18				
			Recessive	CC	3	7	1.31	0.30-5.64	0.72	0.08
				CT+TT	17	52				
D-amino acid oxidase activator (*DAOA*)	rs3916971	T	Genotypic	TT	6	11	-	-	0.20[a]	-
				TC	5	28				
				CC	9	20				
			Allelic	T	17	50	1.01	0.49-2.08	0.99	0.05
				C	23	68				
			Dominant	TT+TC	11	39	0.63	0.22-1.76	0.38	0.14
				CC	9	20				
			Recessive	TT	6	11	1.87	0.59-5.96	0.29	0.22
				TC+CC	14	48				
	rs778293	G	Genotypic	GG	3	14	-	-	0.68[a]	-
				GA	9	22				
				AA	8	22				
			Allelic	G	15	50	0.79	0.38-1.66	0.54	0.09
				A	25	66				
			Dominant	GG+GA	12	36	0.92	0.32-2.59	0.87	0.05
				AA	8	22				
			Recessive	GG	3	14	0.55	0.14-2.18	0.40	0.20
				GA+AA	17	44				
	rs746187	C	Genotypic	CC	3	12	-	-	0.69[a]	-
				CT	11	26				
				TT	6	21				
			Allelic	C	17	50	1.01	0.49-2.08	0.99	0.05
				T	23	68				
			Dominant	CC+CT	14	38	1.29	0.43-3.85	0.65	0.08
				TT	6	21				
			Recessive	CC	3	12	0.69	0.17-2.75	0.60	0.11
				CT+TT	17	47				
Neuregulin 1 (*NRG1*)	rs10503929	C	Genotypic	CC	0	1	-	-	0.74[a]	-
				CT	6	21				
				TT	14	37				
			Allelic	C	6	23	0.73	0.27-1.94	0.53	0.14
				T	34	95				
			Dominant	CC+CT	6	22	0.72	0.24-2.15	0.56	0.10
				TT	14	37				
			Recessive	CC	0	1	0.95	0.04-24.3	0.98	0.05
				CT+TT	20	58				

*p<0.008 (**bold font**); #p<0.05 (*italics*) (significant without Bonferroni correction); [a] Chi-square p-value; [b] power (1-β) calculated using the Fisher's exact test for two independent proportions; OR: Odds ratio; CI: confidence interval for the OR

Supplementary Table S4: Association of *DAO*, *DAOA* and *NRG1* SNPs with APSS (n=98) compared to all other help-seeking group (n=87) at baseline and power analysis

Gene	SNP ID	Minor allele	Model	Genotype/ allele	Phenotype		OR	95% CI	p-value	Power[b]
					APSS (N)	Help-seeking (N)				
D-amino acid oxidase (*DAO*)	rs3918347	G	Genotypic	GG	11	5	-	-	0.053[a]	-
				GA	37	26				
				AA	33	45				
			Allelic	G	59	36	1.84	1.13-3.01	0.01[#]	0.76
				A	103	116				
			Dominant	GG+GA	48	31	2.11	1.12-3.99	0.02[#]	0.63
				AA	33	45				
			Recessive	GG	11	5	2.23	0.74-6.75	0.16	0.69
				GA+AA	70	71				
	rs4623951	C	Genotypic	CC	7	7	-	-	0.99[a]	-
				CT	43	40				
				TT	31	29				
			Allelic	C	57	54	0.99	0.62-1.57	0.95	0.05
				T	105	98				
			Dominant	CC+CT	50	47	1.00	0.52-1.90	0.99	0.05
				TT	31	29				
			Recessive	CC	7	7	0.93	0.31-2.80	0.90	0.06
				CT+TT	74	69				
D-amino acid oxidase activator (*DAOA*)	rs3916971	T	Genotypic	TT	17	19	-	-	0.79[a]	-
				TC	33	32				
				CC	31	26				
			Allelic	T	67	70	0.85	0.54-1.32	0.46	0.11
				C	95	84				
			Dominant	TT+TC	50	51	0.82	0.43-1.58	0.56	0.09
				CC	31	26				
			Recessive	TT	17	19	0.82	0.39-1.71	0.58	0.09
				TC+CC	64	58				
	rs778293	G	Genotypic	GG	17	17	-	-	0.88[a]	-
				GA	34	35				
				AA	29	25				
			Allelic	G	68	69	0.91	0.58-1.42	0.68	0.07
				A	92	85				
			Dominant	GG+GA	51	52	0.85	0.44-1.64	0.62	0.09
				AA	29	25				
			Recessive	GG	17	17	0.95	0.45-2.04	0.90	0.05
				GA+AA	63	60				
	rs746187	C	Genotypic	CC	10	14	-	-	0.57[a]	-
				CT	39	34				
				TT	32	28				
			Allelic	C	59	62	0.83	0.53-1.31	0.43	0.13
				T	103	90				
			Dominant	CC+CT	49	48	0.89	0.47-1.70	0.73	0.07
				TT	32	28				
			Recessive	CC	10	14	0.62	0.26-1.50	0.29	0.33
				CT+TT	71	62				
Neuregulin 1 (*NRG1*)	rs10503929	C	Genotypic	CC	3	1	-	-	0.19[a]	-
				CT	33	23				
				TT	45	53				
			Allelic	C	39	25	1.64	0.93-2.86	0.08	0.60
				T	123	129				
			Dominant	CC+CT	36	24	1.77	0.92-3.39	0.09	0.42
				TT	45	53				
			Recessive	CC	3	1	2.92	0.30-28.7	0.36	0.89
				CT+TT	78	76				

*p<0.008 (**bold font**); [#]p<0.05 (*italics*) (significant without Bonferroni correction); [a] Chi-square p-value; [b] power (1-β) calculated using the Fisher's exact test for two independent proportions; OR: Odds ratio; CI: confidence interval for the OR

Supplementary Table S5: Differences in RDoC domain negative valence systems: threat (acute and sustained) and loss across *DAO*, *DAOA* and *NRG1* SNPs

Gene	SNP ID	Model	Genotypes	Negative valence systems											
				Threat (sum of BAI scale)						Loss (CDSS: item 2)					
				Baseline			LA			Baseline			LA		
				N	Mean± SEM	p-value[a]	N	Mean SEM	p-valuea	N	Mean± SEM	p-value[a]	N	Mean± SEM	p-value[a]
D-amino acid oxidase (*DAO*)	rs3918347	G	GG	16	16.3±1.8	0.48	12	15.8±6.9	0.84	18	1.1±0.2	0.47	14	0.64±0.3	0.73
			GA	57	19.4±1.5		46	11.8±1.5		59	0.97±0.1		48	0.69±0.1	
			AA	67	20.7±1.5		62	11.8±1.5		78	1.1±0.1		67	0.64±0.1	
		D	GG+GA	73	18.71.2	0.42	58	12.7±1.8	0.71	77	0.99±0.1	0.34	62	0.68±0.1	0.62
			AA	67	20.7±1.5		62	11.8±1.5		78	1.1±0.1		67	0.64±0.1	
		R	GG	16	16.3±1.8	0.26	12	15.8±6.9	0.77	18	1.1±0.2	0.72	14	0.64±0.3	0.69
			GA+AA	124	20.1±1.1		108	11.8±1.1		137	1.1±0.1		115	0.66±0.1	
	rs4623951	G	CC	13	24.0±3.6	0.23	15	13.5±3.4	0.5	15	1.1±0.1	0.45	15	0.67±0.2	0.95
			CT	76	20.2±1.3		64	13.6±1.9		81	1.2±0.1		69	0.65±0.1	
			TT	51	17.7±1.5		41	9.5±1.3		59	0.92±0.1		45	0.67±0.1	
		D	CC+CT	89	20.8±1.3	0.16	79	13.6±1.6	0.24	96	1.2±0.1	0.21	84	0.65±0.1	0.77
			TT	51	17.7±1.5		41	9.5±1.3		59	0.92±0.1		45	0.67±0.1	
		R	CC	13	24.0±3.6	0.19	15	13.5±3.4	0.74	15	1.1±0.1	0.62	15	0.67±0.2	0.97
			CT+TT	127	19.2±1.0		105	12.0±1.3		140	1.1±0.1		114	0.66±0.1	
D-amino acid oxidase activator (*DAOA*)	rs3916971	G	TT	31	20.5±2.0	0.68	24	17.3±4.0	0.47	36	1.1±0.2	0.63	31	0.61±0.2	0.65
			TC	62	20.0±1.4		50	11.4±1.6		67	0.97±0.1		52	0.60±0.1	
			CC	47	18.7±1.8		46	10.4±1.3		52	1.2±0.1		46	0.76±0.1	
		D	TT+TC	93	20.2±1.1	0.38	74	13.3±1.7	0.69	103	1.0±0.1	0.40	83	0.60±0.1	0.37
			CC	47	18.7±1.8		46	10.4±1.3		52	1.2±0.1		46	0.76±0.1	
		R	TT	31	20.5±2.0	0.78	24	17.3±4.0	0.22	36	1.1±0.2	0.93	31	0.61±0.2	0.87
			TC+CC	109	19.4±1.1		96	10.9±1.0		119	1.1±0.1		98	0.67±0.1	
	rs778293	G	GG	32	20.9±2.2	0.82	24	18.1±3.9	0.21	35	1.1±0.2	0.35	28	0.89±0.2	0.28
			GA	60	19.1±1.3		49	10.4±1.5		68	0.94±0.1		53	0.51±0.1	
			AA	48	19.6±1.8		47	11.0±1.5		52	1.2±0.1		48	0.69±0.1	
		D	GG+GA	92	19.7±1.2	0.86	73	13.0±1.7	0.66	103	0.99±0.1	0.25	81	0.64±0.1	0.6
			AA	48	19.6±1.8		47	11.0±1.5		52	1.2±0.1		48	0.69±0.1	
		R	GG	32	20.9±2.2	0.54	24	18.1±3.9	0.08	35	1.1±0.2	0.71	28	0.89±0.2	0.24
			GA+AA	108	20.9±2.2		96	10.7±1.1		120	1.1±0.1		101	0.59±0.1	
	rs746187	G	CC	21	19.9±3.2	0.11	20	10.7±2.2	0.38	24	1.4±0.2	*0.04*[#]	21	1.05±0.2	0.11
			CT	64	21.3±1.3		56	13.6±1.9		72	0.88±0.1		61	0.54±0.1	
			TT	55	17.7±1.5		44	11.2±1.9		59	1.2±0.1		47	0.64±0.1	
		D	CC+CT	85	20.9±1.3	0.07	76	12.8±1.5	0.22	96	1.0±0.1	0.40	82	0.67±0.1	0.91
			TT	55	17.7±1.5		44	11.2±1.9		59	1.2±0.1		47	0.64±0.1	
		R	CC	21	19.9±3.2	0.68	20	10.7±2.2	0.86	24	1.4±0.2	0.052	21	1.05±0.2	*0.04*[#]
			CT+TT	119	19.6±1.0		100	12.5±1.3		131	1.0±0.1		108	0.58±0.1	
Neuregulin 1 (*NRG1*)	rs10503929	G	CC	5	14.0±5.6	0.47	4	16.5±7.3	0.53	5	0.6±0.4	0.37	5	0.80±0.6	0.82
			CT	46	20.9±1.8		42	13.4±1.9		51	1.2±0.1		44	0.55±0.1	
			TT	89	19.3±1.2		74	11.3±1.5		99	1.0±0.1		80	0.71±0.1	
		D	CC+CT	51	20.2±1.7	0.81	46	13.7±1.8	0.31	56	1.1±0.1	0.65	49	0.57±0.1	0.54
			TT	89	19.3±1.2		74	11.3±1.5		99	1.0±0.1		80	0.71±0.1	
		R	CC	5	14.0±5.6	0.27	4	16.5±7.3	0.49	5	0.6±0.4	0.23	5	0.80±0.6	0.99
			CT+TT	135	19.9±1.0		116	12.1±1.2		150	1.1±0.1		124	0.65±0.1	

[a] p-value by Kruskal-Wallis test for genotypic model and Mann-Whitney test for dominant and recessive models; *p<0.008 (**bold font**); # 0.008<p<0.05 (*italics*) (significant without Bonferroni correction); G: genotypic model; D: dominant model; R: recessive model; LA: last-available follow-up data until 36 months; BAI: Beck Anxiety Inventory; CDSS: Calgary Depression Rating scale for Schizophrenia; SEM: standard error of the mean

Supplementary Table S6: Differences in RDoC domain cognitive systems: visual and auditory perception across *DAO*, *DAOA* and *NRG1* SNPs

Gene	SNP ID	Model	Genotypes	Cognitive systems											
				Visual perception (SPI-A O4+F1+F2+F3)						Auditory perception (SPI-A O5+F4+F5)					
				Baseline			LA			Baseline			LA		
				N	Mean± SEM	p-value[a]	N	Mean± SEM	p-value[a]	N	Mean± SEM	p-value[a]	N	Mean± SEM	p-value[a]
D-amino acid oxidase (*DAO*)	rs3918347	G	GG	9	3.1±1.4	0.4	8	1.1±0.1	0.31	18	2.3±0.7	0.13	14	1.8±0.7	0.34
			GA	37	3.6±0.5		34	2.4±0.4		52	3.8±0.5		42	2.0±0.4	
			AA	45	3.5±0.6		43	2.3±0.5		72	4.1±0.5		60	2.5±0.4	
		D	GG+GA	46	3.5±0.5	0.57	42	2.1±0.3	0.54	70	3.4±0.4	0.33	56	1.9±0.3	0.27
			AA	45	3.5±0.6		43	2.3±0.5		72	4.1±0.5		60	2.5±0.4	
		R	GG	9	3.1±1.4	0.32	8	1.1±0.1	0.26	18	2.3±0.7	*0.04#*	14	1.8±0.7	0.19
			GA+AA	82	3.6±0.4		77	2.4±0.3		124	4.0±0.4		102	2.3±0.3	
	rs4623951	G	CC	8	5.3±1.3	0.15	9	4.1±1.7	0.30	13	5.2±1.1	0.15	13	3.0±0.9	0.20
			CT	50	3.4±0.5		49	2.2±0.4		76	3.6±0.4		65	2.3±0.3	
			TT	33	3.3±0.6		27	1.7±0.3		53	3.7±0.6		38	1.8±0.4	
		D	CC+CT	58	3.7±0.5	0.44	58	2.5±0.4	0.48	89	3.8±0.4	0.18	78	2.4±0.3	0.13
			TT	33	3.3±0.6		27	1.7±0.3		53	3.7±0.6		38	1.8±0.4	
		R	CC	8	5.3±1.3	0.053	9	4.1±1.7	0.13	13	5.2±1.1	0.09	13	3.0±0.9	0.19
			CT+TT	83	3.4±0.4		76	2.0±0.3		129	3.6±0.3		103	2.1±0.3	
D-amino acid oxidase activator (*DAOA*)	rs3916971	G	TT	26	4.1±0.8	0.09	22	2.9±0.6	*0.02#*	32	3.6±0.5	0.36	26	1.8±0.4	0.96
			TC	35	2.2±0.3		32	1.9±0.5		61	4.2±0.6		46	2.5±0.5	
			CC	30	4.6±0.8		31	2.1±0.5		49	3.3±0.6		44	2.1±0.3	
		D	TT+TC	61	3.0±0.4	0.17	54	2.3±0.4	0.73	93	4.0±0.4	0.15	72	2.3±0.4	0.77
			CC	30	4.6±0.8		31	2.1±0.5		49	3.3±0.6		44	2.1±0.3	
		R	TT	26	4.1±0.8	0.36	22	2.9±0.6	*0.009#*	32	3.6±0.5	0.55	26	1.8±0.4	0.95
			TC+CC	65	3.3±0.4		63	2.0±0.4		110	3.8±0.4		90	2.3±0.3	
	rs778293	G	GG	25	3.9±0.8	0.21	17	1.9±0.4	0.18	32	3.3±0.6	0.93	22	2.1±0.5	0.77
			GA	39	2.9±0.5		35	2.8±0.6		61	3.7±0.5		47	2.0±0.4	
			AA	27	4.0±0.7		33	1.8±0.4		49	4.1±0.6		47	2.5±0.5	
		D	GG+GA	64	3.3±0.4	0.17	52	2.5±0.4	0.07	93	3.6±0.4	0.75	69	2.0±0.3	0.54
			AA	27	4.0±0.7		33	1.8±0.4		49	4.1±0.6		47	2.5±0.5	
		R	GG	25	3.9±0.8	0.62	17	1.9±0.4	0.49	32	3.3±0.6	0.76	22	2.1±0.5	0.91
			GA+AA	66	3.4±0.4		68	2.3±0.4		110	3.9±0.4		94	2.2±0.3	
	rs746187	G	CC	15	2.1±0.6	0.15	15	2.3±0.9	0.95	22	4.2±1.0	0.37	20	3.4±1.0	0.26
			CT	38	3.6±0.6		36	2.0±0.3		67	3.3±0.4		52	2.0±0.3	
			TT	38	4.0±0.6		34	2.4±0.6		53	4.2±0.6		44	2.0±0.3	
		D	CC+CT	53	3.2±0.5	0.27	51	2.1±0.4	0.74	89	3.5±0.4	0.32	72	2.3±0.4	0.89
			TT	38	4.0±0.6		34	2.4±0.6		53	4.2±0.6		44	2.0±0.3	
		R	CC	15	2.1±0.6	0.06	15	2.3±0.9	0.87	22	4.2±1.0	0.55	20	3.4±1.0	0.12
			CT+TT	76	3.8±0.4		70	2.2±0.3		120	3.7±0.4		96	2.0±0.2	
Neuregulin 1 (*NRG1*)	rs10503929	G	CC	2	2.5±1.5	0.64	2	1.0±0.0	0.20	5	1.0±0.5	0.17	4	0.8±0.3	0.30
			CT	28	3.8±0.6		28	2.3±0.4		44	3.8±0.5		41	2.5±0.4	
			TT	61	3.4±0.5		55	2.3±0.4		93	3.9±0.4		71	2.1±0.3	
		D	CC+CT	30	3.7±0.6	0.4	30	2.2±0.4	0.16	49	3.5±0.5	0.80	45	2.4±0.4	0.69
			TT	61	3.4±0.5		55	2.3±0.4		93	3.9±0.4		71	2.1±0.3	
		R	CC	2	2.5±1.5	0.81	2	1.0±0.0	0.40	5	1.0±0.5	0.06	4	0.8±0.3	0.17
			CT+TT	89	3.6±0.4		83	2.3±0.3		137	3.9±0.3		112	2.3±0.3	

[a] p-value by Kruskal-Wallis test for genotypic model and Mann-Whitney test for dominant and recessive models; *p<0.008 (**bold font**); # 0.008<p<0.05 (*italics*) (significant without Bonferroni correction); G: genotypic model; D: dominant model; R: recessive model; LA: last-available follow-up data until 36 months; BAI: Beck Anxiety Inventory; CDSS: Calgary Depression Rating scale for Schizophrenia; SEM: standard error of the mean

Supplementary Table S7: Differences in RDoC domain cognitive system: cognitive control across *DAO*, *DAOA* and *NRG1* SNPs

Gene	SNP ID	Model	Genotypes	Cognitive systems					
				Cognitive control (SIPS: sum of D1-D4)					
				Baseline			LA		
				N	Mean± SEM	p-value[a]	N	Mean± SEM	p-value[a]
D-amino acid oxidase (*DAO*)	rs3918347	G	GG	18	4.8±0.9	0.59	14	2.2±0.5	0.95
			GA	58	4.5±0.4		49	2.4±0.4	
			AA	79	4.1±0.3		65	2.2±0.3	
		D	GG+GA	76	4.6±0.4	0.32	63	2.3±0.3	0.75
			AA	79	4.1±0.3		65	2.2±0.3	
		R	GG	18	4.8±0.9	0.90	14	2.2±0.5	0.85
			GA+AA	137	4.3±0.2		114	2.3±0.2	
	rs4623951	G	CC	15	3.9±0.9	0.65	15	1.9±0.6	0.52
			CT	81	4.4±0.3		69	2.6±0.3	
			TT	59	4.4±0.4		44	2.0±0.3	
		D	CC+CT	96	4.3±0.3	0.56	84	2.4±0.3	0.73
			TT	59	4.4±0.4		44	2.0±0.3	
		R	CC	15	3.9±0.9	0.40	15	1.9±0.6	0.34
			CT+TT	140	4.4±0.3		113	2.3±0.2	
D-amino acid oxidase activator (*DAOA*)	rs3916971	G	TT	36	3.7±0.5	0.43	29	2.6±0.5	0.70
			TC	67	4.6±0.4		53	2.1±0.3	
			CC	52	4.4±0.4		46	2.4±0.4	
		D	TT+TC	103	4.3±0.3	0.67	82	2.2±0.3	0.91
			CC	52	4.4±0.4		46	2.4±0.4	
		R	TT	36	3.7±0.5	0.19	29	2.6±0.5	0.41
			TC+CC	119	4.5±0.3		99	2.2±0.2	
	rs778293	G	GG	35	3.8±0.5	0.49	28	2.7±0.5	0.32
			GA	68	4.7±0.4		52	2.4±0.4	
			AA	52	4.2±0.4		48	1.9±0.3	
		D	GG+GA	103	4.4±0.3	0.90	80	2.5±0.3	0.19
			AA	52	4.2±0.4		48	1.9±0.3	
		R	GG	35	3.8±0.5	0.25	28	2.7±0.5	0.22
			GA+AA	120	4.5±0.3		100	2.2±0.2	
	rs746187	G	CC	24	3.4±0.6	0.22	21	3.0±0.6	0.61
			CT	73	4.6±0.4		59	2.1±0.3	
			TT	58	4.3±0.4		48	2.2±0.4	
		D	CC+CT	97	4.3±0.3	0.60	80	2.4±0.3	0.45
			TT	58	4.3±0.4		48	2.2±0.4	
		R	CC	24	3.4±0.6	0.08	21	3.0±0.6	0.38
			CT+TT	131	4.5±0.3		107	2.2±0.2	
Neuregulin 1 (*NRG1*)	rs10503929	G	CC	5	5.4±2.1	**0.003***	5	2.6±1.0	0.33
			CT	50	5.5±0.5		44	2.5±0.3	
			TT	100	3.7±0.3		79	2.2±0.3	
		D	CC+CT	55	5.5±0.5	**0.001***	49	2.5±0.3	0.14
			TT	100	3.7±0.3		79	2.2±0.3	
		R	CC	5	5.4±2.1	0.71	5	2.6±1.0	0.67
			CT+TT	150	4.3±0.2		123	2.3±0.2	

[a] p-value by Kruskal-Wallis test for genotypic model and Mann-Whitney test for dominant and recessive models; *p<0.008 (**bold font**); # 0.008<p<0.05 (*italics*) (significant without Bonferroni correction); G: genotypic model; D: dominant model; R: recessive model; LA: last-available follow-up data until 36 months; BAI: Beck Anxiety Inventory; CDSS: Calgary Depression Rating scale for Schizophrenia; SEM: standard error of the mean

Supplementary Table S8: Power analysis for RDoC negative valence and cognitive systems across *DAO*, *DAOA*, and *NRG1* SNPs

Gene	SNP ID	Model	Negative valence systems								Cognitive systems											
			Threat (sum of BAI scale)				Loss (CDSS: item 2)				Visual perception (SPI-A O4+F1+F2+F3)				Auditory perception (SPI-A O5+F4+F5)				Cognitive control (SIPS: sum of D1-D4)			
			Baseline		LA		Baseline		LA		Baseline		LA		Baseline		LA		Baseline		LA	
			ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]	ES[a]	P[a]
D-amino acid oxidase (*DAO*)	rs3918347	Genotypic	0.12	0.22	0.09	0.13	0.08	0.14	0.03	0.06	0.04	0.06	0.13	0.16	0.15	0.33	0.10	0.14	0.08	0.13	0.03	0.06
		Dominant	0.18	0.18	0.61	0.92	0.16	0.16	0.05	0.06	0.003	0.05	0.07	0.06	0.17	0.17	0.2	0.18	0.14	0.14	0.03	0.05
		Recessive	0.33	0.23	0.31	0.18	0	0.05	0.02	0.05	0.13	0.07	0.43	0.21	0.44	0.41	0.17	0.09	0.18	0.11	0.04	0.05
	rs4623951	Genotypic	0.21	0.76	0.15	0.29	0.12	0.25	0.01	0.05	0.16	0.24	0.24	0.49	0.11	0.20	0.12	0.20	0.05	0.08	0.12	0.22
		Dominant	0.26	0.32	0.32	0.38	0.26	0.34	0.02	0.05	0.11	0.08	0.26	0.19	0.02	0.05	0.2	0.17	0.04	0.06	0.18	0.16
		Recessive	0.40	0.78	0.11	0.07	0.09	0.06	0.01	0.05	0.55	0.31	0.76	0.57	0.38	0.26	0.32	0.19	0.17	0.10	0.20	0.11
D-amino acid oxidase activator (*DAOA*)	rs3916971	Genotypic	0.06	0.09	0.20	0.49	0.10	0.17	0.09	0.13	0.31	0.75	0.15	0.22	0.11	0.18	0.09	0.13	0.13	0.26	0.09	0.13
		Dominant	0.13	0.11	0.23	0.22	0.19	0.20	0.18	0.17	0.45	0.52	0.07	0.06	0.18	0.18	0.05	0.06	0.03	0.05	0.05	0.06
		Recessive	0.09	0.07	0.50	0.59	0	0.05	0.07	0.06	0.22	0.16	0.34	0.28	0.05	0.06	0.17	0.12	0.29	0.33	0.16	0.12
	rs778293	Genotypic	0.06	0.09	0.23	0.61	0.12	0.25	0.17	0.37	0.15	0.23	0.18	0.28	0.08	0.11	0.08	0.11	0.12	0.25	0.12	0.22
		Dominant	0.006	0.05	0.15	0.12	0.22	0.25	0.06	0.06	0.21	0.15	0.28	0.23	0.14	0.12	0.16	0.13	0.07	0.07	0.24	0.26
		Recessive	0.14	0.11	0.58	0.72	0.04	0.06	0.35	0.37	0.16	0.10	0.13	0.08	0.14	0.11	0.03	0.05	0.23	0.22	0.2	0.16
	rs746187	Genotypic	0.14	0.31	0.10	0.15	0.21	0.62	0.21	0.54	0.19	0.32	0.07	0.08	0.12	0.21	0.19	0.42	0.14	0.31	0.12	0.21
		Dominant	0.28	0.37	0.12	0.10	0.17	0.17	0.03	0.05	0.22	0.17	0.12	0.08	0.17	0.17	0.13	0.11	0.003	0.05	0.07	0.06
		Recessive	0.02	0.05	0.15	0.09	0.43	0.48	0.55	0.62	0.49	0.40	0.01	0.05	0.13	0.09	0.51	0.56	0.12	0.08	0.33	0.27
Neuregulin 1 (*NRG1*)	rs10503929	Genotypic	0.11	0.20	0.10	0.14	0.12	0.26	0.09	0.14	0.06	0.08	0.07	0.08	0.13	0.28	0.13	0.20	0.29	0.91	0.07	0.09
		Dominant	0.08	0.07	0.18	0.16	0.12	0.11	0.16	0.14	0.07	0.06	0.04	0.05	0.10	0.08	0.10	0.08	0.59	0.99	0.13	0.11
		Recessive	0.51	0.20	0.34	0.10	0.52	0.21	0.17	0.07	0.30	0.07	0.45	0.09	0.73	0.36	0.55	0.19	0.36	0.12	0.13	0.06

[a] ANOVA (for 3 groups) and t-test for 2 groups; LA: last-available follow-up data until 24 months; BAI: Beck Anxiety Inventory; CDSS: Calgary Depression Rating scale for Schizophrenia; SPI-A: Schizophrenia Proneness Instrument-Adult version ($\geq$ 18 years); SPI-CY: Schizophrenia Proneness Instrument-Child and Youth version ($<$ 18 years); SIPS: Structured Interview for Prodromal Symptoms; ES: effect size; P: Power; SEM: standard error of the mean

Supplementary Table S9: Correlation between *NRG1* gene expression and RDoC domains at baseline and 36 months

RDoC domains	RDoC construct (scale)	Time-point	*NRG1* gene expression		
			N	Spearmans rank correlation co-efficient	p-value
Negative valence systems	Threat (sum of BAI scale)	Baseline	168	0.133	0.085
		LA	135	0.049	0.575
	Loss (CDSS: item 2)	Baseline	181	-0.057	0.444
		LA	146	0.196	**0.018***
Cognitive systems	Visual perception (SPI-A O4+F1+F2+F3)	Baseline	114	-0.012	0.901
		LA	103	-0.099	0.318
	Auditory perception (SPI-A O5+F4+F5)	Baseline	167	0.004	0.963
		LA	132	-0.046	0.598
	Cognitive control (SIPS: sum of D1-D4)	Baseline	182	0.051	0.491
		LA	145	0.106	0.203

*p<0.05 (**bold font**); LA: last-available follow-up data until 36 months; RDoC: Research Domain Criteria; BAI: Beck Anxiety Inventory; CDSS: Calgary Depression Rating scale for Schizophrenia; SPI-A: Schizophrenia Proneness Instrument-Adult version (≥18 years); SPI-CY: Schizophrenia Proneness Instrument-Child and Youth version (<18 years); SIPS: Structured Interview for Prodromal Symptoms

Supplementary Table S10: *NRG1* gene expression in peripheral whole blood across different phenotypes and power analysis

Clinical phenotypes	*NRG1* gene expression			Effect size[a]	Power[a]
	N	Mean±SEM	Mann-Whitney p-value		
Converters	23	1.41±0.27	0.892	0.16	0.10
Non-converters	58	1.25±0.11			
APSS	80	1.36±0.12	0.650	0.16	0.17
All other help-seeking	76	1.21±0.09			

*p<0.05 (**bold font**); [a] t-test; APSS: attenuated positive symptoms syndrome; HR: high risk for psychosis; UHR: ultra-high risk for psychosis

Supplementary Table S11: *NRG1* gene expression in peripheral whole blood across *NRG1* SNP genotypes and models

NRG1 SNP ID	*NRG1* mRNA expression											
	Genotypes	N	Mean± SEM	K-W p-value	Dom model	N	Mean± SEM	M-W p-value	Rec model	N	Mean± SEM	M-W p-value
rs10503929	CC	7	0.99± 0.15	0.975	CC+ CT	67	1.25± 0.12	0.848	CC	7	0.99± 0.15	0.868
	CT	60	1.28± 0.14						CT+ TT	165	1.22± 0.07	
	TT	105	1.19± 0.07		TT	105	1.19± 0.07					

*p<0.05 (**bold font**); K-W p-value: Kruskal-Wallis p-value; M-W p-value: Mann-Whitney p-value; Dom model: dominant model; Rec model: recessive model

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5.4 Study IV: Controversial effects of D-amino acid oxidase activator (DAOA)/G72 on D-amino acid oxidase (DAO) activity in human neuronal, astrocyte, and kidney cell lines: The N-methyl D-aspartate (NMDA) receptor hypofunction point of view

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Author contributions

VJ and EG designed the experiments. VJ performed *in vitro* experiments and analyzed data. ZFB performed *in silico* experiments and analyzed data. VJ drafted and revised the manuscript. MP, SW, and EG reviewed the manuscript. All authors have approved the final manuscript.

5.4.1 Abstract

Dysfunction of D-amino acid oxidase (*DAO*) and DAO activator (*DAOA*)/*G72* genes have been linked to neuropsychiatric disorders. The glutamate hypothesis of schizophrenia has proposed that increased DAO activity leads to decreased D-serine, which subsequently may lead to N-methyl-D-aspartate (NMDA) receptor hypofunction. It has been shown that DAOA binds to DAO and increases its activity. However, there are also studies showing DAOA decreases DAO activity. Thus, the effect of DAOA on DAO is controversial. We aimed to understand the effect of DAOA on DAO activity in neuron-like (SH-SY5Y), astrocyte-like (1321N1), and kidney-like (HEK293) human cell lines. DAO activity was measured based on the release of hydrogen peroxide and its interaction with Amplex Red reagent. We found that DAOA increases DAO activity only in HEK293 cells, but has no effect on DAO activity in SH-SY5Y and 1321N1 cells. This might be because of different signaling pathways, or due to lower DAO and DAOA expression in SH-SY5Y and 1321N1 cells compared to HEK293 cells, but also due to different compartmentalization of the proteins. The lower DAO and DAOA expression in neuron-like SH-SY5Y and astrocyte-like 1321N1 cells might be due to tightly regulated expression, as previously reported in the human post-mortem brain. Our simulation experiments to demonstrate the interaction between DAOA and human DAO (hDAO) showed that hDAO holoenzyme [hDAO with flavine adenine dinucleotide (FAD)] becomes more flexible and misfolded in the presence of DAOA, whereas DAOA had no effect on hDAO apoprotein (hDAO without FAD), which indicate that DAOA inactivates hDAO holoenzyme. Furthermore, patch-clamp analysis demonstrated no effect of DAOA on NMDA receptor activity in NR1/NR2A HEK293 cells. In summary, the interaction between DAO and DAOA seems to be cell type and its biochemical characteristics dependent which still needs to be elucidated.

5.4.2 Introduction

The human D-amino acid oxidase (*DAO*/*DAAO*) gene is located at chromosome 12q24, and encodes for a ∼39 kDa protein of 347 amino acids (Verrall et al., 2010). The human DAO activator (*DAOA*)/*G72* gene is a primate specific gene located at chromosome 13q33, and encodes for a ∼20 kDa protein of 153 amino acids (Benzel et al., 2008). Previous studies have shown evidence for significant association of nucleotide variations at *DAO* and *DAOA* locus with schizophrenia and bipolar disorder (Detera-Wadleigh and McMahon, 2006; Allen et al., 2008; Prata et al., 2008; Gatt et al., 2015). Although the effects of these *DAO* and *DAOA* nucleotide variations on their mRNA and protein expression in schizophrenia is not yet studied, these genes still remain as candidate genes for schizophrenia because of their role in the glutamatergic signaling.

DAO is a peroxisomal flavoenzyme. It catalyzes the oxidation of D-amino acids through concomitant reduction of flavine adenine dinucleotide (FAD), producing corresponding imino acid, which is then hydrolyzed to yield ammonia and corresponding α-keto acid. During FAD reoxidation, hydrogen peroxide is produced (Verrall et al., 2010). FAD binding is weaker in human DAO (hDAO) compared to DAO from other species, which provides hDAO a potential means to regulate DAO activity (Caldinelli et al., 2009). DAO protein and enzymatic activity is present mainly in the human kidney, liver, and brain (Sasabe et al., 2014; Uhlén et al., 2015; Jagannath et al., 2017b). In the human brain, its main substrate is D-serine (Pollegioni et al., 2007; Sacchi et al., 2012). D-serine serves as a co-agonist at the glycine site of the N-methyl-D-aspartate (NMDA) receptor. NMDA receptors are glutamate ionotropic receptors which require both glutamate and co-agonist (D-serine or glycine) to function normally (Panatier et al., 2006; Henneberger et al., 2010; Papouin et al., 2012). Thus, DAO can regulate the function of NMDA receptors via D-serine breakdown.

The glutamate hypothesis of schizophrenia is based on the NMDA receptor hypofunction (Stahl et al., 2007). One possible explanation for NMDA receptor hypofunction theory proposed in schizophrenia is increased DAO activity leading to decreased D-serine which subsequently causes hypofunction of the NMDA receptors. Chumakov and colleagues showed that DAOA binds to DAO and increases its activity (Chumakov et al., 2002). However, Sacchi and colleagues showed that DAOA binds to DAO and decreases its activity (Sacchi et al., 2008). Furthermore, Kvajo and colleagues showed that there was no interaction between DAO and DAOA (Kvajo et al., 2008). Thus, the effect of DAOA on DAO is controversial, and yet to be elucidated. Previous *in vitro* studies have shown that DAOA localizes in mitochondria and causes

mitochondrial dysfunction (Kvajo et al., 2008; Sacchi et al., 2011; Otte et al., 2014). Thus, the exact function of DAOA is not yet completely understood. Since the microscopic interactions between DAO and DAOA may play an additional role in DAO activation, *in silico* molecular dynamics simulations may contribute in understanding the role of DAOA on DAO activity. Thus, this approach may contribute to the *in vitro* insight into the nature and interactions between these two proteins. In particular, we performed simulations of different DAO (apoprotein, holoenzyme) forms with and without DAOA, and studied the stability of DAO in terms of its flexibility, thus verifying one of the above DAO and DAOA interaction hypotheses.

DAO and DAOA proteins are detected in the human brain with brain region specificity which is tightly regulated (Jagannath et al., 2017b). DAO has been shown to interact with DAOA in glial cells (Sacchi et al., 2016). However, DAO is not solely glial, but it has been reported to be also expressed in neurons (Verrall et al., 2010). In our study, we used neuron-like SH-SY5Y, astrocyte-like 1321N1, and kidney-like HEK293 cells to understand the interaction between DAO and DAOA proteins. We overexpressed DAO and DAOA in these human cell lines because of the lower endogenous expression of *DAO* and *DAOA* mRNA in these cells (Uhlén et al., 2015; Rouillard et al., 2016).

Since the interaction between DAO and DAOA is still not clear, we aimed (1) to understand the effect of DAOA on DAO activity in different types of human cell lines and (2) to understand the effect of DAOA on NMDA receptor activity in human cell lines.

5.4.3 Materials and methods

Cell culture

The human embryonic kidney HEK293 cell line (85120602, Sigma-Aldrich) and human astrocytoma 1321N1 cell line (86030402, Sigma-Aldrich) were cultured in Dulbecco's Modified Eagle Medium (DMEM; 41966029, ThermoFisher scientific) containing 10% fetal bovine serum (FBS; 10270106, ThermoFisher scientific), and incubated at 37°C in a humidified atmosphere of 5% CO_2. The human neuroblastoma SH-SY5Y cell line (CRL-2266, ATCC) was cultured in a 1:1 mixture of DMEM and DMEM/F12 (D8437, Sigma-Aldrich) supplemented with 10% FBS, and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Transient transfection

The human LG72 cDNA (GenBank sequence: AY138546) cloned into the pEGFPN1 vector was a generous gift from the Institute of Molecular Psychiatry, University of Bonn, Germany (Otte et al., 2011), and the pEGFPN1 construct (Takara Clontech) was used as a control plasmid. The human DAO cDNA (GenBank sequence: BC029057) cloned into pCMV3-c-Myc vector (HG13372-CM, Sino Biological) and the pCMV3-c-Myc vector (CV014, Sino Biological) were used for transfection. For all experiments except cell viability, HEK293, 1321N1, and SH-SY5Y cells were seeded into 6-well plates (3335, Corning) at a density of 2x10^5 cells, 5x10^5, and 1x10^6 cells in 1 mL growth medium, respectively. The cells were allowed to adhere for 24 hours. The cells were transfected with 5 μg of above-mentioned plasmids using Xfect transfection reagent (631317, Takara Clontech) according to manufacturer's guidelines. The growth medium was exchanged 4 hours after transfection. For all experiments, cells were incubated for 48 hours following transfection.

DAO activity assay

In order to evaluate whether DAOA affects DAO activity, DAO activity was determined based on the estimation of hydrogen peroxide formation as previously described (Sikka et al., 2010). The transfected cells were harvested with 0.05% Trypsin-EDTA (25300054, ThermoFisher Scientific) solution, transferred to a 15 mL falcon tube, and centrifuged for 5 minutes at 12,000 xg. The cell pellets were homogenized in 100 μL of cold sodium phosphate buffer (50 mM Na_2HPO_4, pH 7.4) with 5 mm stainless steel beads (69989, Qiagen) using the TissueLyser II (Qiagen). In a 384-well optical-bottom plate (142761, ThermoFisher Scientific), 10 μL of homogenate was added to a solution of 100 μM Amplex red (A36006, ThermoFisher Scientific), 0.25 U/mL horseradish peroxidase (10108090001, Sigma-Aldrich), 50 mM D-serine (S4250,

Sigma-Aldrich), and with or without 10 μM FAD (F6625, Sigma-Aldrich) in a total volume of 20 μl of sodium phosphate buffer. After 1 hour of incubation at 37°C, the fluorescence was measured with a Mithras^2 LB 943 Multimode Reader (Berthold technologies) using 544 nm excitation (slit 22 nm) and 590 nm emission (slit 20 nm) filters. On each plate, we included 3 controls namely, a control without cell homogenate, a control without D-serine, and a control inhibiting DAO activity with a DAO inhibitor, 100 μM 6-methyl-benzo[d]isoxazol-3-ol (027-640-512, MolPort). The fluorescence values of cell homogenates were calculated by subtracting their fluorescence values from fluorescence values of control without D-serine. As the wells transfected with control plasmids (pCMV3-c-Myc and pEGFPN1) showed fluorescence similar to the control without D-serine, fluorescence values of cells transfected with LG72 and DAO plasmids were used for analysis.

Cell viability assay

We evaluated cell viability to make sure that the results obtained for DAO activity were not biased by the sensitivity to different cell lines to transfection. The viability of transfected cells was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (G3582, Promega) according to manufacturer's guidelines. HEK293, 1321N1, and SH-SY5Y cells were seeded into 96-well plates (83.3925, Sarstedt) at a density of 2x10^4 cells, 5x10^4, and 1x10^5 cells in 100 μL growth medium, respectively. They were allowed to adhere for 24 hours and were transfected with 1.2 μg of plasmids using Xfect transfection reagent (631317, Takara Clontech) according to manufacturers guidelines. The growth medium was exchanged 4 hours after transfection. Each transfection condition was analyzed in triplicates. The cells were incubated for 48 hours following transfection. Then, 20 μl of CellTiter 96® AQueous One Solution Reagent was added to each well and incubated at 37°C for 4 hours. The absorbance was measured at 490 nm using Mithras^2 LB 943 Multimode Reader (Berthold technologies). The absorbance values were corrected by subtracting the average absorbance from the control wells with no cells.

Simulation setup for hDAO and DAOA interactions

In this work, Molecular Dynamics (MD) simulations were performed. All MD simulations were carried out using the GROMACS software (Abraham et al., 2015). In all simulations, the AMBER-14SB and the TIP3P forcefields (Jorgensen et al., 1983; Maier et al., 2015) were used for the protein and water, respectively. The simulated conditions were ambient, at a constant temperature of 300 K, pressure of 1 atm and concentration (NPT ensemble). The simulated

systems were: hDAO without FAD in water, FAD/hDAO complex in water, FAD/hDAO/DAOA complex in water, and hDAO/DAOA complex in water.

hDAO without FAD (apoprotein): The hDAO monomer structure was extracted from the dimer protein data bank (PDB) structure of hDAO (PDB: 2E48), and the FAD ligand was removed.

FAD/hDAO (holoenzyme) complex: The structure of this complex was obtained by extracting the hDAO monomer structure from the dimer PDB structure of hDAO while retaining the FAD ligand. The topology of the complex was obtained using the AMBER tools and the AMBER-14SB forcefield, after having created the FAD topology. The FAD topology was obtained using the gaff forcefield, followed by a geometry optimization using Gaussian package and the B3LYP/6-31G, constructing the electrostatic potential surface and assigning charges to atoms using RESP (Bayly et al., 1993).

FAD/hDAO/DAOA complex: The structure of this complex was obtained by using the patchdock docking server (Schneidman-Duhovny et al., 2005), and adding constraints on the dimer interface so that DAOA residues 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153 were within 0.4 nm of any amino acids within 1 nm of hDAO amino acids 37, 138, 163, 185, 188, 191, 193, 194, 195, 196. These contacts have been found to occur in the hDAO-DAOA complex (Chang et al., 2013). As mentioned earlier, building the FAD topology and using AMBER tools to combine the topology of FAD, hDAO and G72 protein, the final complexes topology was obtained. Simulations were performed using the structure of the best binding pose.

hDAO/DAOA complex: The procedure for constructing the structure of this complex was the same as the above ones, except the topology of the complex was obtained by using the AMBER tools for complex's structure.

DAOA protein: Since there was no available crystal structure for the human DAOA protein, we obtained its structure by using homology modelling of the protein sequence with accession number AAN08432. The homology modelling was performed using the I-TAISER server (Yang and Zhang, 2015). The best predicted homolog structure was used as the DAOA protein.

NMDA receptor currents using whole-cell patch clamp recording

HEK293 cells stably expressing NMDA receptor subunits NR1 and NR2A (NR1/NR2A HEK293 cells; B'SYS, Switzerland) were used. They were cultured in DMEM/F12 (D8437, Sigma-Aldrich) supplemented with 10% FBS (RNBF7902, ThermoFisher scientific), 1% penicillin/streptomycin (10378016,

ThermoFisher scientific), 100 μg/mL hygromycin (10687010, ThermoFisher scientific), 15 μg/mL blasticidin (A1113903, ThermoFisher scientific), 1 μg/mL puromycin (A1113803, ThermoFisher scientific), and incubated at 37°C in a humidified atmosphere of 5% CO_2. The cells (2x10^5 cells/well) were seeded onto Poly-L-Lysine (P4832, Sigma-Aldrich) coated coverslips placed in 6-well plates (92412, TPP) and were allowed to adhere for 24 hours. The cells were transfected with 5 μg of LG72 and control pEGFPN1 plasmids using Xfect transfection reagent according to manufacturer's guidelines. The growth medium was exchanged 4 hours after transfection. Expression of NR1 and NR2A subunits in the cells were induced 24 hours after transfection using 2.5 μg/mL tetracycline (T7660, Sigma-Aldrich) in the presence of 50 μM AP5 (ab120003, abcam). The whole-cell patch clamp recording was performed 48 hours after transfection at room temperature using Amplifier EPC-10 and Preamplifier EPC-10 (both, HEKA Electronics). For recording, the coverslips with transfected or non-transfected cells were placed in a 35-mm culture dish containing 2 mL of standard bath solution (137 mM sodium chloride, 4 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, 10 mM HEPES, 10 mM D-Glucose, pH 7.4 adjusted with sodium hydroxide). The transfected cells were identified visually by green florescent protein (GFP) fluorescence signal using inverted microscope IM (Zeiss). The patch pipettes were filled with pipette solution (130 mM potassium chloride, 1 mM magnesium chloride, 5 mM magnesium-ATP, 10 mM HEPES, 5 mM EGTA, pH 7.2 adjusted with potassium hydroxide). After formation of Gigaohm seal between the patch pipettes and single cell (pipette resistance range: 2.5 MΩ to 6.0 MΩ; seal resistance range: $>$1 GΩ), the cell membrane across the pipette tip was ruptured to ensure electrical access to the cell interior (whole-cell patch configuration). In case of poor seal quality, the process of seal formation was repeated with a different cell and a new pipette. As soon as a stable seal was established, magnesium-free bath solution (137 mM sodium chloride, 4 mM potassium chloride, 2.8 mM calcium chloride, 10 mM HEPES, 10 mM D-Glucose, 0.02% Cremophor, pH 7.4 adjusted with sodium hydroxide) was perfused and NMDA inward currents were measured upon application of submaximal concentrations of NMDA (100 μM; M3262, Sigma-Aldrich)/Glycine (5 μM; 410225, Sigma-Aldrich) to patch-clamped cells for 4 seconds. If the current was too unstable for measurement, another cell was recorded. During the entire experiment, the membrane potential of the cell was clamped at -80 mV. We used only data from cells treated with the complete application protocol for analysis. At least 5 cells were recorded per condition and three independent experiments were performed. Data was acquired using PatchMaster (HEKA Electronics, version v2x73_2) software and analyzed using

Microsoft Excel 2003 software. The NMDA peak current density was calculated by dividing means of peak current amplitudes (pA) from at least 5 cells by cell capacitance (pF). The NMDA mean current density was calculated by dividing means of steady state current amplitudes (pA) from at least 5 cells by cell capacitance (pF).

Statistical analysis

The results presented in this study are from at least three independent experiments. IBM® SPSS® Statistics (version 23) software was used for statistical analysis. Shapiro-Wilk test with Lilliefors significance correction was used to assess the normality of the distribution of NMDA receptor current, DAO activity and cell viability data. As the NMDA receptor current data was normally distributed, we used one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test to analyze the difference between the groups, and $p < 0.05$ was considered statistically significant. As the DAO activity and cell viability data was also normally distributed, we used independent-samples t-test to analyze the difference between two groups, and $p < 0.05$ was considered statistically significant. GraphPad Prism software (version 6.01) was used to plot the graphs.

5.4.4 Results

DAO activity and cell viability in SH-SY5Y cells

To understand the effect of DAOA on DAO activity, SH-SY5Y cells were transfected with either DAO plasmid alone or both DAO and G72 plasmids. To achieve DAO-activity signal in SH-SY5Y cells, the addition of 10 μM FAD was necessary. There were no significant differences in DAO activity between the two transfection conditions, namely DAO and G72+DAO (Figure 1A). In order to verify that the non-significant differences in DAO activity between single (DAO) and double transfection (G72+DAO) conditions is not due to cell death, we performed cell viability assay. There were no significant differences in cell viability between the two transfection conditions (Figure 1B).

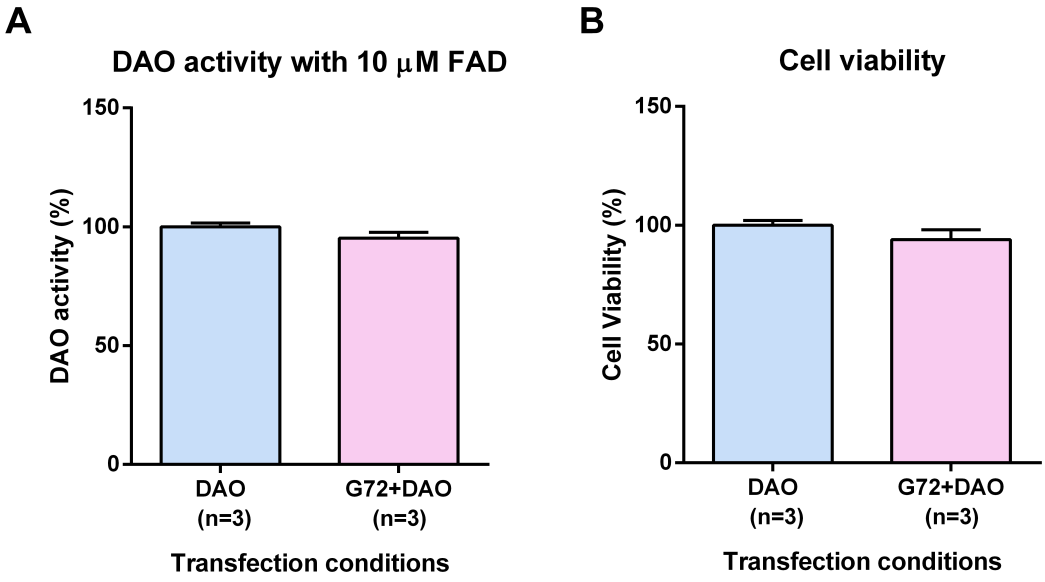


Figure 1: DAO activity and cell viability in SH-SY5Y cells. (**A**) DAO activity with the addition of 10 μM FAD across two transfection conditions: DAO and G72+DAO. (**B**) Cell viability across two transfection conditions: DAO and G72+DAO. Data is presented as bar graphs with mean±SEM. Differences between the two transfection conditions was assessed by the independent-samples t-test (*$p<0.05$).

DAO activity and cell viability in 1321N1 cells

To verify the results obtained in SH-SY5Y neuroblastoma cells with neuron-like phenotype, we retested effects of DAOA on DAO activity in the human 1321N1 cells which have an astrocyte-like phenotype. Again, we single and double transfected 1321N1 cells with DAO plasmid and both DAO and G72 plasmids, respectively. In contrast to SH-SY5Y cells, DAO activity assay in 1321N1 cells worked both with and without addition of 10 μM FAD. We did not find any significant differences in DAO activity without (Figure 2A) and with (Figure 2B)

addition of 10 μM FAD between the two transfection conditions as well as no significant differences in cell viability between the two transfection conditions (Figure 2C).

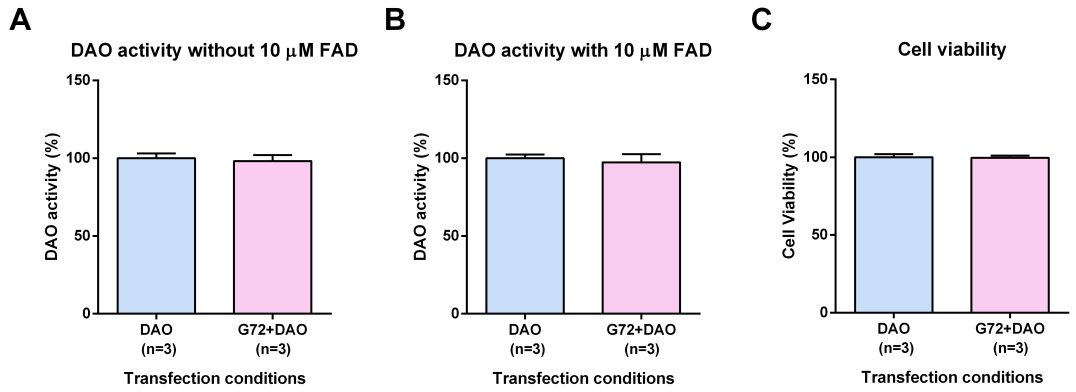


Figure 2: DAO activity and cell viability in 1321N1 cells. (**A**) DAO activity without the addition of 10 μM FAD across two transfection conditions: DAO and G72+DAO. (**B**) DAO activity with the addition of 10 μM FAD across two transfection conditions: DAO and G72+DAO. (**C**) Cell viability across two transfection conditions: DAO and G72+DAO. Data is presented as bar graphs with mean±SEM. Differences between the two transfection conditions was assessed by the independent-samples t-test (*$p<0.05$).

DAO activity and cell viability in HEK293 cells

To test the tissue related specificity of the results obtained in neuron-glia like cell lines, we determined the effect of DAOA on DAO activity also in human HEK293 cells with kidney epithelium-like phenotype. HEK293 cells were single and double transfected with DAO plasmid and with both DAO and G72 plasmids, respectively to understand the effect of DAOA on DAO activity. Similar to 1321N1 cells, DAO activity assay in HEK293 cells worked with and without addition of 10 μM FAD. In contrast to SH-SY5Y and 1321N1 cells, DAO activity without ($t=3.024$, $df=10$, $p=0.013$; Figure 3A) and with ($t=5.305$, $df=10$, $p=0.0003$; Figure 3B) addition of 10 μM FAD was significantly increased in double transfected HEK293 cells (G72+DAO) compared to the single transfected (DAO) HEK293 cells. The cell viability assay was performed to confirm that the differences in DAO activity are not due to cell death. There were no significant differences in cell viability between the two transfection conditions (Figure 3C).

Although DAO protein was detected at the expected size of 40 kDa in pEGFPN1+pCMV3-c-Myc and pCMV3-c-Myc transfection conditions in all three cell lines (data not shown), we could not detect DAO activity signal in these transfection conditions which might be because the endogenous DAO enzyme is in FAD unbound inactive state.

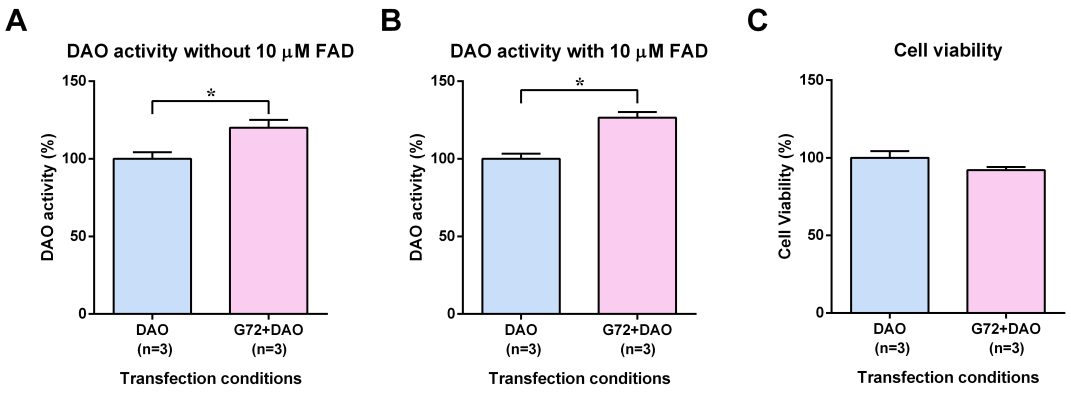


Figure 3: DAO activity and cell viability in HEK293 cells. (**A**) DAO activity without the addition of 10 μM FAD across two transfection conditions: DAO and G72+DAO. (**B**) DAO activity with the addition of 10 μM FAD across two transfection conditions: DAO and G72+DAO. (**C**) Cell viability across two transfection conditions: DAO and G72+DAO. Data is presented as bar graphs with mean$\pm$SEM. Differences between the two transfection conditions was assessed by the independent-samples t-test (*$p<0.05$).

In silico interactions between hDAO and DAOA

In order to understand the effect of DAOA protein on the holoenzyme (FAD/hDAO) and apoprotein (hDAO without FAD) form of the hDAO, the fluctuations of the Cα atoms of each amino acid were determined. We found that there was a slight decrease in FAD/hDAO complex flexibility compared to hDAO without FAD (Figure 4A). Our simulations show that upon FAD/hDAO/DAOA complex formation, FAD/hDAO become much more flexible (Figure 4B) and misfolded (Figure 4C) than FAD/hDAO complex without DAOA. Contrarily, the apoprotein form of the hDAO when in complex with DAOA, shows much less flexibility (Figure 4B), and retains its folded structure (Figure 4D).

NMDA receptor currents in NR1/NR2A HEK293 cells

To understand the effect of DAOA on NMDA receptor activity, NR1/NR2A HEK293 cells were transfected with G72 plasmid. There were no significant differences in mean NMDA receptor current (Supplementary Figure S1A) or in peak NMDA receptor current (Supplementary Figure S1B) between all three conditions namely, G72 transfected, pEGFPN1 plasmid transfected and non-transfected.

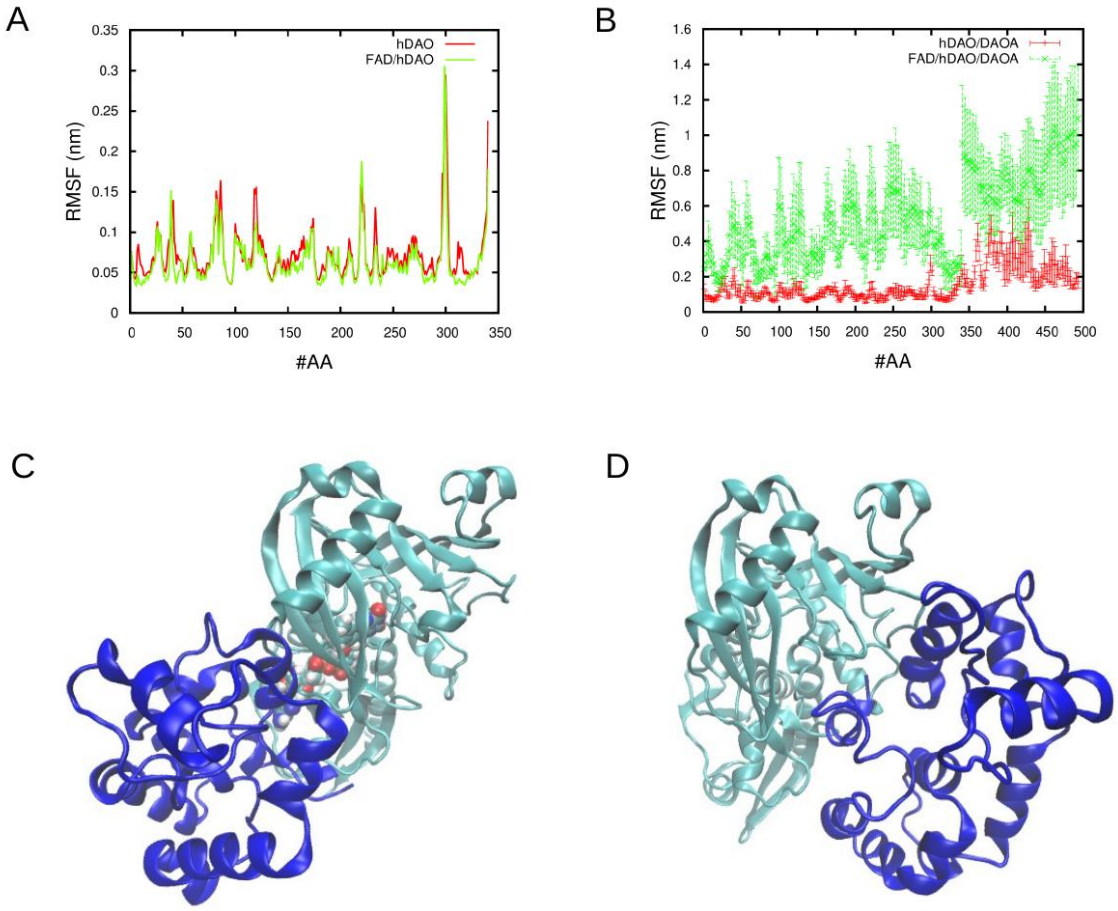


Figure 4: Fluctuations and 3D conformations of hDAO holoenzyme and apoprotein complex with DAOA. Room mean square fluctuations of the Cα atom per amino acid (#AA) of (**A**) the hDAO without FAD (red), and the FAD/hDAO complex (green), and (**B**) the hDAO/DAOA complex (red) and FAD/hDAO/DAOA complex (green). Note that the amino acid index 0-340 corresponds to the hDAO Cα atoms, whereas the index 341-494 corresponds to the Cα atoms of DAOA amino acids. 3D conformations taken from Molecular Dynamics simulation of holoenzyme FAD/hDAO/DAOA complex (**C**) and apoprotein hDAO/DAOA complex (**D**) with hDAO in green colour and DAOA in blue colour.

DAO and *DAOA* mRNA expression in transfected SH-SY5Y, 1321N1, and HEK293 cells

In order to verify and compare the expression of endogenous and overexpressed *DAO* and *DAOA* mRNA in transfected SH-SY5Y, 1321N1, and HEK293 cells, we measured the *DAO* and *DAOA* mRNA levels using qRT-PCR. We found that there were no significant differences in *DAO* mRNA levels between the double transfection (G72+DAO) and single transfection (DAO) conditions in SH-SY5Y and 1321N1 cells, but a significantly lower endogenous *DAO* mRNA levels in pEGFPN1+pCMV3-c-Myc and pCMV3-c-Myc transfected compared to the G72+DAO and DAO transfected SH-SY5Y ($F=74.91$, $df=3$, $p<0.0001$) and 1321N1 cells ($F=135.98$, $df=3$, $p<0.0001$; Figure 5A). There was a significant decrease in *DAO* mRNA levels in double transfected (G72+DAO) compared to the single

transfected (DAO) HEK293 cells (Bonferroni test p=0.001), and endogenous *DAO* mRNA levels were significantly lower in pEGFPN1+pCMV3-c-Myc and pCMV3-c-Myc transfected HEK293 cells compared to G72+DAO and DAO transfected HEK293 cells (F=182.58, df=3, p<0.0001; Figure 5A). We found that the endogenous *DAOA* mRNA levels were significantly lower in DAO, pEGFPN1+pCMV3-c-Myc, pCMV3-c-Myc transfected than G72+DAO transfected SH-SY5Y (F=3656.23, df=3, p<0.0001), 1321N1 (F=112.39, df=3, p<0.0001), and HEK293 cells (F=858.86, df=3, p<0.0001; Figure 5B). There were significant differences in *DAO* mRNA levels between the three cell lines in G72+DAO transfection condition (F=213.76, df=2, p<0.0001; Figure 5C), and *DAO* mRNA levels were the highest in HEK293 cells compared to the 1321N1 and SH-SY5Y cells in DAO transfection condition (F=222.68, df=2, p<0.0001; Figure 5D). We found that there were significant differences in *DAOA* mRNA levels between the three cell lines both in G72+DAO (F=164.83, df=2, p<0.0001; Figure 5E) and DAO (F=56.35, df=2, p<0.0001; Figure 5F) transfection conditions. *DAOA* mRNA levels were the highest in HEK293 cells followed by intermediate levels in 1321N1 cells and low levels in SH-SY5Y cells.

DAO and DAOA protein expression in transfected SH-SY5Y, 1321N1, and HEK293 cells

To verify and compare the overexpression of DAO and DAOA proteins in transfected SH-SY5Y, 1321N1, and HEK293 cells, we determined DAO and DAOA proteins using western blot. DAO protein was detected in all three cell lines at the expected size of 40 kDa in G72+DAO (Figure 6A) and DAO (Figure 6E) transfection conditions. DAO protein levels were the highest in 1321N1 cells compared to HEK293 and SH-SY5Y cells in G72+DAO (Figure 6B) and DAO (Figure 6F) transfection conditions. We detected c-Myc-DAO fusion protein in all three cell lines at the expected size of 41 kDa in G72+DAO (Figure 6C) and DAO (Figure 6G) transfection conditions. HEK293 cells had highest c-Myc-DAO fusion protein levels compared to 1321N1 and SH-SY5Y cells in G72+DAO (Figure 6D) and DAO (Figure 6H) transfection conditions. DAOA protein was detected in all three cell lines at the expected size of 18 kDa in G72+DAO (Figure 6I) and DAO (Figure 6M) transfection conditions. DAOA protein levels were the highest in HEK293 cells compared to 1321N1 and SH-SY5Y cells in G72+DAO (Figure 6J) and DAO (Figure 6N) transfection conditions. GFP-DAOA fusion protein was detected in all three cell lines at the expected size of 45 kDa in the double transfection with G72+DAO (Figure 6K), but not in the single transfection with DAO (Figure 6O and 6P). GFP-DAOA fusion protein levels were the highest in HEK293 cells compared to 1321N1 and

SH-SY5Y cells in G72+DAO (Figure 6L) transfection condition.

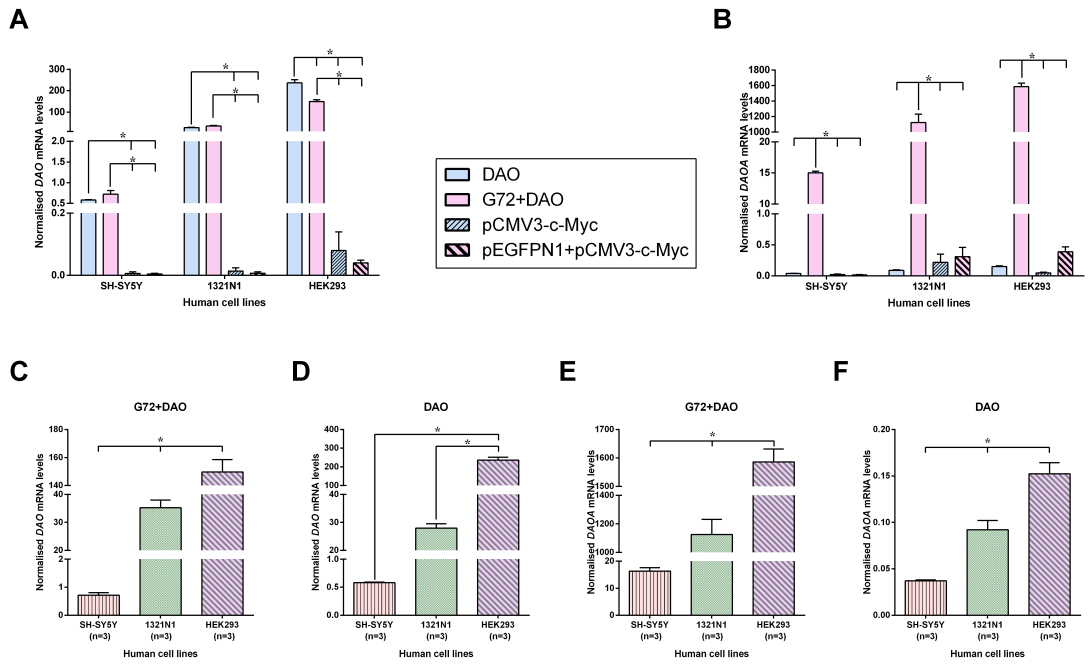


Figure 5: *DAO* and *DAOA* mRNA levels in SH-SY5Y, 1321N1 and HEK293 cells across different transfection conditions. *DAO* (**A**) and *DAOA* (**B**) mRNA levels across four transfection conditions: DAO, G72+DAO, pCMV3-c-Myc, and pEGFPN1+ pCMV3-c-Myc in all three cell lines. *DAO* mRNA levels in two transfection conditions: G72+DAO (**C**) and DAO (**D**). *DAOA* mRNA levels in two transfection conditions: G72+DAO (**E**) and DAO (**F**). Data is presented as bar graphs with mean±SEM. Differences in *DAO* and *DAOA* mRNA levels between four transfection conditions, and also between the three cell lines was assessed by one-way ANOVA followed by post-hoc Bonferroni test (*$p<0.05$).

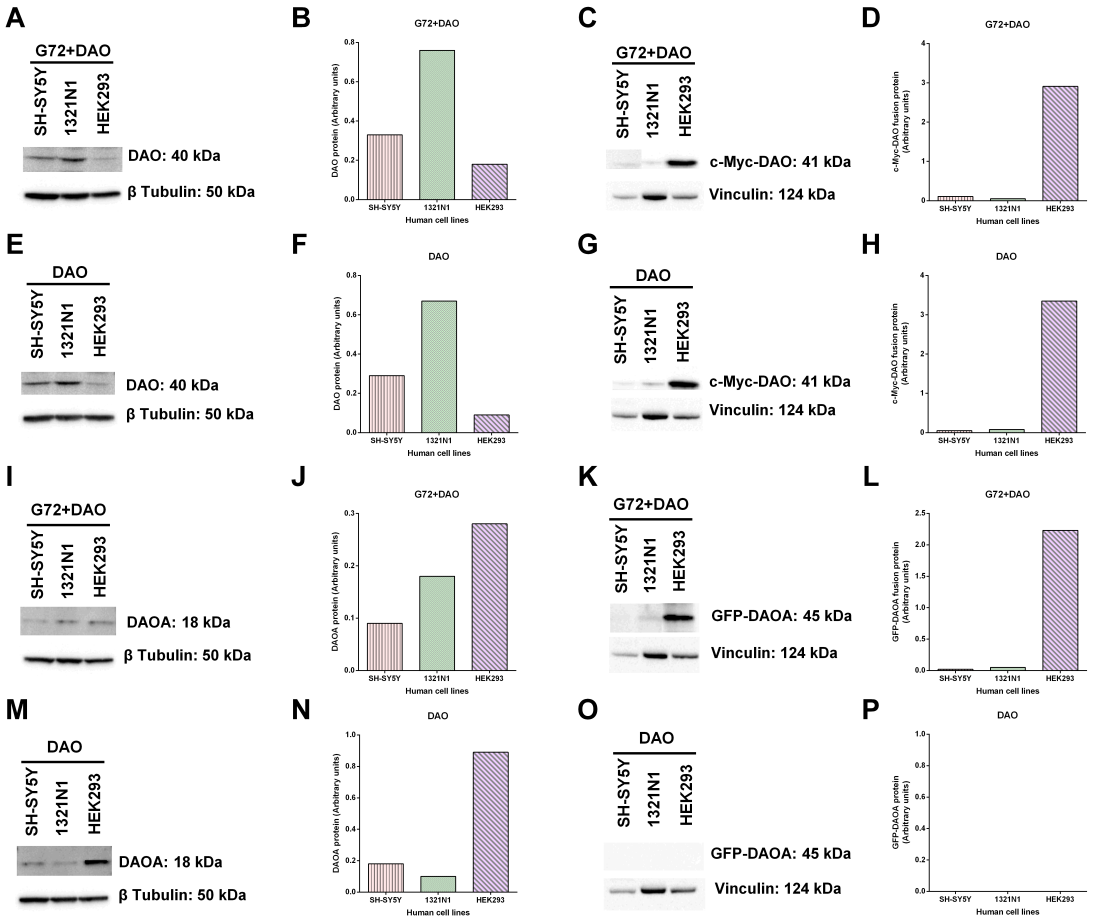


Figure 6: DAO and DAOA protein levels in SH-SY5Y, 1321N1 and HEK293 cells. Representative western blot images for DAO protein expression levels in three cell lines across two transfection conditions: G72+DAO (**A**) and DAO (**E**), and DAO protein detected at an expected size of 40 kDa. DAO protein levels (arbitrary units) in three cells lines across two transfection conditions: G72+DAO (**B**) and DAO (**F**). Representative western blot images for c-Myc-DAO fusion protein expression levels in three cell lines across two transfection conditions: G72+DAO (**C**) and DAO (**G**), and c-Myc-DAO fusion protein detected at an expected size of 41 kDa. c-Myc-DAO protein levels (arbitrary units) in three cells lines across two transfection conditions: G72+DAO (**D**) and DAO (**H**). Representative western blot images for DAOA protein expression levels in three cell lines across two transfection conditions: G72+DAO (**I**) and DAO (**M**), and DAOA protein detected at an expected size of 18 kDa. DAOA protein levels (arbitrary units) in three cells lines across two transfection conditions: G72+DAO (**J**) and DAO (**N**). Representative western blot images for GFP-DAOA fusion protein expression levels in three cell lines across two transfection conditions: G72+DAO (**K**) and DAO (**O**), and GFP-DAOA fusion protein detected at an expected size of 45 kDa. GFP-DAOA protein levels (arbitrary units) in three cells lines across two transfection conditions: G72+DAO (**L**) and DAO (**P**).

Table 1: Summary of publications to date showing effect of DAOA on DAO activity

Serial No.	Publications	Species of DAO protein	Species of DAOA protein	Recombinant proteins or types of cell lines used	Substrate used	DAO activity assay method used	FAD added (+) or not (-)	Enzymatic reaction incubation temperature	Effect of DAOA on DAO activity
1	(Chumakov et al., 2002)	Porcine	Human	*DAOA* cDNA was expressed in *E. coli* BL21(DE3) cells using the pET11b expression vector, and recombinant DAOA protein was isolated using chromatography, and DAO protein was purified from Sigma crude preparation	D-serine	o-dianisidine	+	30°C	Increases DAO activity
2	(Chang et al., 2013)	Human	Human	*DAO* and *DAOA* cDNA were expressed in *E. coli* BL21(DE3)pLysS cells using the pET23a expression vector, and recombinant DAO and DAOA proteins were isolated using HisTrap FF column and column-refolding procedure (Molla et al., 2006a), respectively	D-alanine	o-phenylenediamine	-	25°C	Increases DAO activity
3	(Birolo et al., 2016)	Human	Human	*DAO* and *DAOA* cDNA were expressed in *E. coli* BL21(DE3) cells using the pET11b expression vector, and recombinant DAO and DAOA proteins were isolated using anionic exchange chromatography on Sepharose FF column	D-alanine	Oxygen electrode	+	25°C	Decreases DAO activity
4	(Sacchi et al., 2008)	Human	Human	Human glioblastoma U87 cell line	D-serine	Amplex Red	+	Room temperature	Decreases DAO activity
5	(Kvajo et al., 2008)	Human	Human	Human glioblastoma U251 cell line	D-proline	Alexa-coupled tyramide	-	Room temperature	No effect on DAO activity
6	Current study	Human	Human	Human neuroblastoma SH-SY5Y cell line	D-serine	Amplex Red	+	37°C	No effect on DAO activity
				Human astrocytoma 1321N1 cell line	D-serine	Amplex Red	+/-	37°C	No effect on DAO activity
				Human embryonic kidney HEK293 cell line	D-serine	Amplex Red	+/-	37°C	Increases DAO activity

5.4.5 Discussion

DAO and *DAOA* genes are alleged to be involved in pathophysiology of neuropsychiatric disorders such as schizophrenia and bipolar disorder (Detera-Wadleigh and McMahon, 2006; Allen et al., 2008; Prata et al., 2008; Gatt et al., 2015), but the interactions between these genes remains unclear to date. There are several lines of evidence in humans suggesting NMDA receptor hypofunction in the pathophysiology of schizophrenia. Healthy controls manifested primary symptoms of schizophrenia on administration of NMDA receptor antagonists such as phencyclidine and ketamine (Anis et al., 1983; Krystal et al., 1994). Patients with NMDA receptor specific antibodies were reported to be associated with severe psychosis (Dalmau et al., 2008). A study revealed reduced expression of obligatory NMDA receptor subunit NR1 in post-mortem brains of schizophrenia patients (Weickert et al., 2013), and reduced D-serine in cerebrospinal fluid of schizophrenia patients (Hashimoto et al., 2005). Studies have also shown increased DAO activity in brains of schizophrenia patients compared to the healthy controls (Kapoor et al., 2006; Burnet et al., 2008; Madeira et al., 2008). There is only one published study using single-photon emission computed tomography (SPECT) with NMDA receptor tracer [^{123}I]CNS-1261 which showed lower NMDA receptor binding in left hippocampus of schizophrenia patients compared to healthy controls (Pilowsky et al., 2006), however this finding awaits confirmation. Although, the aforementioned studies provide evidence for NMDA receptor hypofunction in schizophrenia, it is still not clear what NMDA receptor hypofunction means at the molecular level. At present there are no drugs on the market to treat the negative symptoms and cognitive deficits of schizophrenia, and clinical trials with DAO inhibitors or D-serine have not shown a conclusive or strong effect in schizophrenia (Smith et al., 2010). Thus, there is an urgent need for new drugs for the treatment of negative symptoms and cognitive deficits of schizophrenia, which might be achieved by understanding the interaction between DAO and DAOA, and their subsequent effect on NMDA receptors. In this study, we aimed to understand the interaction between DAO and DAOA by determining the effect of DAOA on DAO and NMDA receptor activity in different types of human cell lines.

We chose three human cell lines namely, neuron-like SH-SY5Y, astrocyte-like 1321N1 cells, and kidney-like HEK293 cells to verify and compare the effects of DAOA on DAO activity. We found that DAOA increases the activity of DAO only in HEK293 cells, but not in the SH-SY5Y and 1321N1 cells. One possible reason for this result might be because of different signaling pathways

in kidney-like HEK293 cells versus neuron-like SH-SY5Y and astrocyte-like 1321N1 cells. Moreover, our previous post-mortem study (Jagannath et al., 2017b) showed that there is a tight transcriptional regulation in DAO and DAOA expression in the human brain during development and aging, which might also be the reason that we found no effect of DAOA on DAO activity in the neuron-like SH-SY5Y and astrocyte-like 1321N1 cells. Chumakov and colleagues showed that increasing concentrations of recombinant human DAOA protein causes porcine DAO activation (Chumakov et al., 2002). In the current study, we found that HEK293 cells overexpressed *DAOA* mRNA significantly more than in SH-SY5Y and 1321N1 cells, which might explain why DAO activity increased only in HEK293 cells when co-transfected with DAOA. A study conducted using recombinant hDAO and DAOA proteins isolated from *E.coli* found that DAOA increases DAO activity (Chang et al., 2013), but a recent study using recombinant hDAO and DAOA proteins isolated from *E.coli* found that DAOA decreases DAO activity (Birolo et al., 2016). Moreover, another study found that DAOA even decreases DAO activity in human glioblastoma U87 cell line (Sacchi et al., 2008). However, following the last result, a study conducted in human glioblastoma U251 cell line, reported that DAOA has no effect on DAO activity (Kvajo et al., 2008). All these studies performed the DAO activity assay based on the detection of hydrogen peroxide released from oxidation of D-amino acids by the DAO enzyme. The differences in results found in these studies might be because of substrate, cell lines, and enzymatic reaction temperature used (see summary in Table 1). Chumakov and colleagues used recombinant human DAOA but recombinant porcine DAO to perform DAO activity assay with D-serine as substrate and the enzymatic reaction was performed at 30°C (Chumakov et al., 2002). However, two independent research groups used D-alanine as substrate and the assay was performed at 25°C (Chang et al., 2013; Birolo et al., 2016). Furthermore, another two groups conducted the DAO activity assay at room temperature, but Kvajo and colleagues used D-proline as substrate (Kvajo et al., 2008), while Sacchi and colleagues used D-serine as substrate (Sacchi et al., 2008). In our study, we used D-serine as substrate and performed the assay at 37°C, which corresponds to the human body temperature. The DAO activity assay conducted with D-proline as substrate (Kvajo et al., 2008) can be biased because D-proline is also a substrate of human D-aspartate oxidase (Katane et al., 2015). A study found that DAO shows lower catalytic efficiency and substrate affinity on the physiological substrate D-serine compared to D-alanine (Molla et al., 2006b), which might explain the discrepancies found between studies using these substrates. We found that DAO activity assay worked in 1321N1 and HEK293 cells even without the addition

of 10 μM FAD, which might be because of endogenous production of FAD by these cells. Indeed, FAD co-factor binding was reported to be essential for the DAO enzyme activity (Molla et al., 2006b). Nevertheless, we found that addition of 10 μM FAD had no effect on the results obtained comparing to those obtained without the addition of 10 μM FAD in these two cell lines. In studies using recombinant DAO and DAOA proteins, two of the studies, which did not use FAD in DAO activity assay, found DAOA to be a DAO activator (Chumakov et al., 2002; Chang et al., 2013). However, a recent study with human recombinant DAO and DAOA proteins, where they added FAD in DAO activity assay found DAOA to be a DAO inhibitor (Birolo et al., 2016). Thus, additional studies in a more realistic conditions i.e., human induced pluripotent stem cells (hiPSC) derived neurons and glia using gene editing tools will be required to conclusively determine whether DAOA is a DAO activator or inhibitor.

In our simulation experiments, we found that there is a slight decrease in FAD/hDAO (holoenzyme) complex flexibility compared to the hDAO without FAD (apoprotein) which indicates that the hDAO holoenzyme is more stable than the hDAO apoprotein. This result coincides with the previous spectroscopic evidence of hDAO holoenzyme having slightly higher melting temperature than the hDAO apoprotein (Caldinelli et al., 2009; Sacchi et al., 2012). Previous studies based on spectroscopy and proteolysis experiments have shown that DAOA changes the tertiary structure of FAD/hDAO and leads to decrease in hDAO holoenzyme form (Caldinelli et al., 2010), which is in line with our simulations that showed DAOA makes FAD/hDAO more flexible (i.e., less stable) and misfolded. However, our simulations showed that DAOA had no effect on hDAO apoprotein structure which corroborates with previous proteolysis experiments that showed hDAO apoprotein structure is not affected by DAOA (Caldinelli et al., 2010). The difference in flexibility of hDAO apoprotein and holoenzyme in complex with DAOA can be explained by the different binding positions of DAOA. DAOA binds to hDAO holoenzyme much closer to the FAD binding pocket, which causes an increase in the complex's fluctuations (i.e., less stable), compared to the hDAO apoprotein where DAOA binds farther away from the FAD binding pocket. Thus, our simulations indicate that DAOA inactivates hDAO holoenzyme and not the hDAO apoprotein. However, we did not observe the similar inactivating effect of DAOA on DAO activity in our cell lines which might be due to the fact that in our simulations we used only hDAO and DAOA proteins in an artificial system. Moreover, as indicated in our previous postmortem study (Jagannath et al., 2017b), the tight regulation of DAO and DAOA in the human brain compared to the periphery might

be an additional reason for the *in vitro* differences found in neuron-like and astrocyte-like cells compared to the kidney-like cells.

Accordingly, going one-step forward, we tested whether DAOA overexpression modulates NMDA receptor activity using induced NR1/NR2A HEK293 cells. In this study, we did not find an effect of DAOA on NMDA receptor currents in NR1/NR2A HEK293 cell line, despite our results showing DAOA increases DAO activity in HEK293 cells. This might be because of several reasons namely; the endogenous DAO (binding partner of DAOA) in NR1/NR2A HEK293 cell line is not in an active form leading to no effect on D-serine, unavailability of D-serine transporters in these NR1/NR2A HEK293 cells to transport the D-serine extracellularly for its action on NMDA receptors, and the DAOA transfected NR1/NR2A HEK293 cell line is an artificial model system which doesn't recapitulate the human tripartite synapse and the complex signaling pathways of human neurons and glia. A study conducted in primary cultures from rat hippocampus using whole cell patch clamp technique showed that DAO inhibitors lead to increase in NMDA receptor mediated currents (Strick et al., 2011). To our knowledge, there are no papers showing the effect of DAOA on NMDA receptor currents *in vitro*. Thus, future experiments must be conducted, probably also in a more complex cellular system, to understand the effect of DAOA on NMDA receptors.

In summary, we found that DAOA increases DAO activity only in kidney-like HEK293, but not in neuron-like SH-SY5Y and astrocyte-like 1321N1 cells. We also found no effect of DAOA on NMDA receptor currents in NR1/NR2A HEK293 cells. As our study was performed using human cell lines, in order to confirm the tight regulation of DAO and DAOA assumed in the human central nervous system (CNS), modelling the interactions between DAO and DAOA proteins in schizophrenia patient specific neuroglial culture i.e., hiPSC derived neurons and glia co-cultures and comparing them to healthy control derived neuroglial cultures might further help in settling the debate of DAOA effect on DAO and NMDA receptor activity..

5.4.6 Supplementary Information

Supplementary Methods

S1 RNA isolation, cDNA synthesis, and quantification of *DAO* and *DAOA* mRNA levels using qRT-PCR

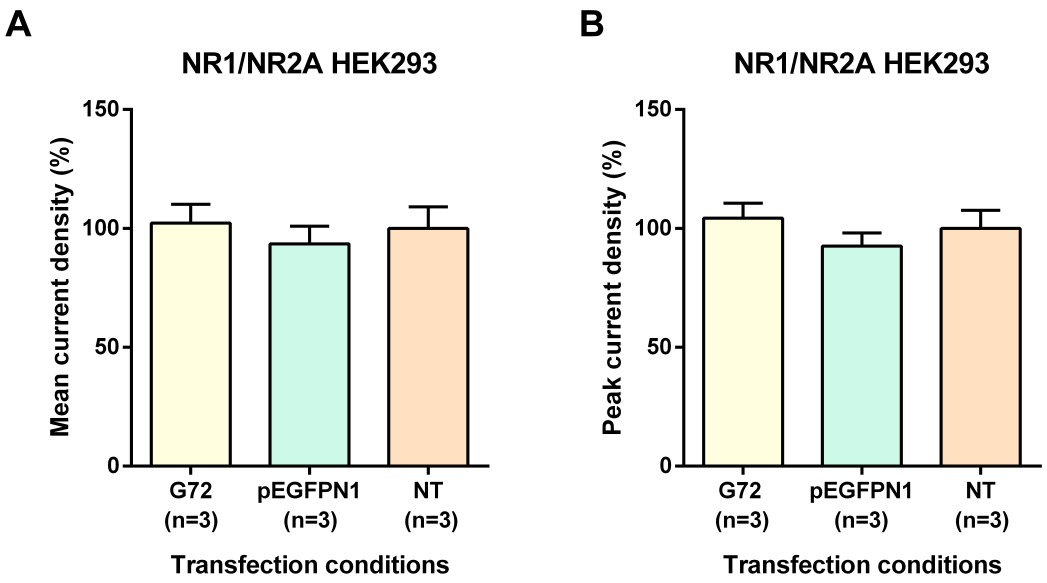
RNA was isolated from transfected SH-SY5Y, 1321N1, and HEK293 cells using RNeasy Plus Mini Kit (74136, Qiagen) according to manufacturer's guidelines. Firstly, the transfected cells were lysed directly in 6-well plates, and were disrupted and homogenized in the lysis buffer provided in the kit using the TissueLyser II (Qiagen). Secondly, the homogenized lysates were added to the gDNA Eliminator spin column to remove the genomic DNA. Finally, the lysates were added to the RNeasy spin column and RNA was eluted. We used a spectrophotometer (NanoVue Plus, GE Healthcare Life Sciences) to measure RNA concentrations, A260/A280, and A260/A230 ratios. We reverse transcribed 500 ng of RNA using iScript^{TM} cDNA Synthesis Kit (Bio-Rad) as per manufacturer's protocol. In a subset of samples, negative controls were prepared with RNA without reverse transcriptase enzyme. Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed using cDNA, QuantiFast SYBR Green PCR kit (Qiagen), 1 μM DAO or DAOA primers, and reference genes [β-actin (*ACTB*; QT01680476), ribosomal protein L13a (*RPL13A*; QT00089915), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; QT01192646), peptidylprolyl isomerase A (*PPIA*; QT00866137), and ribosomal RNA (*R18S*; QT00019936); all from Qiagen]. The DAOA primer (QT00058863) was purchased from Qiagen, and the DAO primer was purchased from Microsynth, the sequence of which has been described by (Verrall et al., 2007) (forward primer: CGCAGACGTGATTGTCAACT; reverse primer: GGATGATGTACGGGGAATTG). *DAO* and *DAOA* mRNA levels were normalized to the reference genes. LinRegPCR program was used to calculate the PCR efficiencies (Ruijter et al., 2009), and mean PCR efficiencies for all studied amplicons were 91-92%. Normalized *DAO* and *DAOA* mRNA levels were quantified using qBASE plus software (Biogazelle) as described in detail previously (Jagannath et al., 2017b).

S2 Protein isolation, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Protein was isolated from transfected SH-SY5Y, 1321N1, and HEK293 cells using radioimmunoprecipitation assay buffer (RIPA; R0278, Sigma-Aldrich) supplemented with protease inhibitor (04693124001, Sigma-Aldrich) and phosphatase inhibitor (04906845001, Sigma-Aldrich) as per manufacturer's

guidelines. The transfected cells were washed twice with ice-cold 1x phosphate buffered saline (PBS) pH 7.4 (10010015, ThermoFisher Scientific). The cells were scraped and collected in RIPA buffer, and were incubated for 30 minutes at 4°C with constant agitation. The cell lysates were centrifuged at 12000 rpm for 10 minutes at 4°C, and supernatants were collected. The protein concentration was determined using the Bradford assay (B6916, Sigma-Aldrich)(Bradford, 1976). Protein lysates (16 μg) were mixed with NuPAGE® LDS Sample Buffer (NP0007, ThermoFisher Scientific) and NuPAGE® Sample Reducing Agent (NP0009, ThermoFisher Scientific), and heated at 70°C for 10 minutes. Then, the lysates were loaded onto NuPAGE^{TM} Novex^{TM} 4-12% Bis-Tris Protein Gels (NP0322BOX, ThermoFisher Scientific), and were subjected to electrophoresis at 180 V for 1 hour in XCell SureLock® Mini-Cell (ThermoFisher Scientific). The gels were transferred onto nitrocellulose membranes (IB301002, ThermoFisher Scientific) using iBlot® Gel Transfer Device (ThermoFisher Scientific). The membranes were blocked using 5% non-fat milk (T145.1, Roth) in 1x PBS pH 7.4 with 0.1% Tween-20 (P9416, Sigma-Aldrich) (PBST) for 1 hour at room temperature (RT). After that, the membranes were incubated with primary antibodies against DAO (1:500; ab196563, abcam) or DAOA (1:100; ab205502, abcam) or c-Myc (1:1000; ab32, abcam) or GFP (1:1000; ab1218, abcam) or -tubulin (1:1000; ab6046, abcam) or vinculin (1:10000; ab129002, abcam) diluted in 5% non-fat milk-PBST at 4°C overnight. The membranes were washed thrice with PBST, and then incubated with anti-rabbit secondary HRP-conjugated antibody (1:2000; ab97051, abcam) or anti-mouse secondary HRP-conjugated antibody (1:2000; ab6789, abcam) for 1 hour at RT. The protein bands were visualized with 20X LumiGLO® Reagent and 20X Peroxide (7003P, Cell Signaling) using ChemiDoc^{TM} XRS+ System with Image Lab^{TM} software (Bio-Rad). The molecular weight of the detected bands was estimated using Novex® Sharp Pre-stained Protein Standard (LC5800, ThermoFisher Scientific). The protein bands were quantified and were normalized to loading controls (β-tubulin or vinculin) using the ImageJ software (Schneider et al., 2012).

Supplementary Figures



Supplementary Figure S1: NMDA receptor currents in NR1/NR2A HEK293 cells. (**A**) Mean NMDA receptor current across three transfections conditions: G72, pEGFPN1 plasmid, and non-transfected (NT). (**B**) Peak NMDA receptor current across three transfections conditions: G72, pEGFPN1 plasmid, and non-transfected (NT). Data is presented as bar graphs with mean±SEM. Differences between the three transfection conditions was assessed by one-way ANOVA (*$p<0.05$).

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6. General Discussion

The overall objectives of this thesis were to elucidate the importance of *DAO* and *DAOA* genes in neurodevelopment, the potential of these gene polymorphisms to emerge as predictive markers for schizophrenia, and the interaction between these genes. These objectives were achieved by investigating these two genes of interest using different model systems, namely, spatiotemporal expression studies of these genes in normal human post-mortem brains, genetic risk and endophenotype analysis in at-risk for psychosis population, and molecular interactions between DAO and DAOA in human cell lines. First, the results and conclusions of each study will be summarised, and then their implications in the broader context will be discussed.

In the post-mortem study (study I), *DAO* mRNA levels were found to be higher in the cerebellum, but DAO protein levels were found to be lower in cerebellum compared to other brain regions studied (brainstem, amygdala, striatum, thalamus, frontal cortex), which might suggest post-transcriptional regulation [e.g., microRNA (miRNA)]. Furthermore, DAOA protein was detected in all brain regions studied, but *DAOA* mRNA was not detected in these brain regions, which might be suggestive of tightly regulated DAOA expression or extremely localized expression or post-transcriptional regulation (e.g., RNA methylation). We found that there was a significant positive correlation of *DAO* mRNA levels with age till 2 years in cerebellum and amygdala, which might highlight the importance of this gene during brain development. There was a significant positive correlation between DAO and DAOA protein levels in all brain regions studied except frontal cortex, which suggests simultaneous expression of these proteins, but with this finding, we cannot comment on interactions between these proteins. There was no significant effect of *DAO* and *DAOA* SNPs on their expression. Moreover, *in silico* DNA methylation results showed that *DAO* and *DAOA* CpG sites in the cerebellum were significantly more methylated than in the frontal cortex, which might explain the brain-region specific differential DAO and DAOA expression. However, we could not correlate the DNA methylation levels at *DAO* and *DAOA* CpG sites with DAO and DAOA expression levels because we did not measure the DNA methylation in our samples.

In the meta-analysis study (study II), we confirmed that 1 *DAO* SNP (rs4623951) out of 8 *DAO* SNPs studied, and 2 *DAOA* SNPs (rs778293 and rs3916971) out of 12 *DAOA* SNPs studied were found to be significantly associated with schizophrenia. The C-allele of *DAO* rs4623951 was found to be protective

against schizophrenia across all pooled studies [Odds ratio (OR)=0.88, 95% confidence interval (CI)=0.79-0.98, p=0.02, N=3,143)]. The G-allele of *DAOA* rs778293 was to be a risk allele for schizophrenia in Asian patients (OR=1.17, 95% CI=1.08-1.27, p=0.00008, N=6,117), and the T-allele of *DAOA* rs3916971 was found to be protective against schizophrenia across all pooled studies (OR=0.84, 95% CI=0.73-0.96, p=0.01, N=1,765). In all these three significant SNPs, there were no new eligible studies since SzGene meta-analysis (Allen et al., 2008) to be included in our meta-analysis.

In the at-risk for psychosis study (study III), *DAO* rs3918347 GA+AA genotype carriers experienced nominally more auditory perception disturbances (RDoC cognitive system: auditory perception) than GG genotype carriers (G-allele: risk for schizophrenia). We found that *DAOA* rs746187 CC genotype carriers experienced nominally more hopelessness (RDoC negative valence system: loss) than CT+TT genotype carriers (T-allele: risk for schizophrenia), and *DAOA* rs3916971 TT genotype carriers experienced nominally more visual perception disturbances (RDoC cognitive system: visual perception) than TC+CC genotype carriers (T-allele: protective against schizophrenia). These results suggest that *DAO* and *DAOA* SNPs might emerge as predictive markers for endophenotypes such as hopelessness and perception disturbances in at-risk for psychosis individuals, only after replication and validation in future studies. Furthermore, *DAO* and *DAOA* SNPs did not emerge as predictive markers for conversion to schizophrenia-spectrum disorders in at-risk for psychosis individuals at 36 months follow-up. We found that the schizophrenia risk G-allele of *DAO* rs3918347 nominally increased the risk for those ultra-high risk (UHR) for psychosis individuals with attenuated positive symptoms syndrome (APSS). We could not detect *DAO* and *DAOA* mRNA levels in the whole blood of at-risk for psychosis individuals.

In the cell culture study (study IV), we found that DAOA increased DAO activity only in human kidney-like HEK293 cells, but DAOA had no effect on DAO activity in human neuron-like SH-SY5Y and human astrocyte-like 1321N1 cells. This might be because of different signalling pathways, different compartmentalization of DAO and DAOA proteins and lower DAO and DAOA overexpressed proteins in 1321N1 and SH-SY5Y cells compared to HEK293 cells. We also found that DAOA had no effect on NMDA receptor activity in HEK293 cells stably transfected with NMDA receptor subunits NR1 and NR2A (NR1/NR2A HEK293). This might be because of reasons such as (i) the endogenous DAO protein might be in an inactive form in NR1/NR2A HEK293 cells leading to no effect on D-serine, (ii) unavailability of D-serine transporters

in these cells, and (iii) these cells are a simple model that do not recapitulate the tripartite synapse and its complex signalling. The simulation experiments showed that DAOA makes human DAO (hDAO) holoenzyme (hDAO with FAD) more flexible and misfolded than hDAO apoprotein (hDAO without FAD), which might suggest that DAOA inactivates hDAO. This *in silico* inactivating effect of DAOA on DAO was not observed in the cell lines studied, which might be because the simulations are an artificial system.

DAO and DAOA in neurodevelopment and their dysregulation in schizophrenia

Human neurodevelopment is a complex and precisely regulated process that transpires over an extended period of time, during which both structural and functional changes occur. This process depends on the precise regulation of gene and protein expression (Kang et al., 2011; Tebbenkamp et al., 2014). Inherited genetic information, inherited epigenetic marks, and environmental influences converge at the level of transcriptome that results in cell-type specific, tissue-specific, spatial and temporal patterns of expression of genes to guide neurodevelopment. Thus, the transcriptome represents a complex interplay between genetics and epigenetics, which makes transcriptome studies valuable as they provide insight into the complex process such as human brain development (Ziats et al., 2015). In order to understand how putative genes can contribute to a developmental disorder like schizophrenia, it is important to study the normal expression pattern and functions of these genes in different brain regions during the developmental time window of interest. The human brain transcriptome has unique characteristics such as expression of higher levels of mRNA transcripts than other species, expression of around 85% of all genes encoded in the human genome at some point during development, increased alternative splicing and brain-specific transcript expression, abundance of non-coding RNAs, and brain region-specific expression (Ziats et al., 2015). We studied the trajectories of DAO and DAOA expression in the normal human post-mortem brain from prenatal age to 91 years, which showed that expression of these genes are age and brain-region specific and these genes are highly regulated at the level of transcription, which might suggest that these genes are important for neurodevelopment. Dysregulation of these genes during neurodevelopment might lead to brain-region specific functional and structural abnormalities, which might manifest later as schizophrenia.

A post-mortem study reported an increase in DAO mRNA and activity in the cerebellum, but no change in *DAO* mRNA in the cerebral cortex of schizophrenia patients compared to the healthy controls (Kapoor et al., 2006). These cortical and

cerebellar mRNA findings were replicated in a subsequent study (Verrall et al., 2007), and DAO immunoreactivity showed trend increase in the cerebellum, but could not be quantified in the prefrontal cortex of schizophrenia patients compared to healthy controls. A subsequent study confirmed the increase in *DAO* mRNA and DAO activity in the cerebellum of schizophrenia patients (Burnet et al., 2008). A post-mortem study found no change in DAO protein levels in the hippocampus and prefrontal cortex of schizophrenia patients (Bendikov et al., 2007), however, a study found an increase in DAO activity in the parietal cortex of schizophrenia patients compared to healthy controls (Madeira et al., 2008). Overall, these data provide clear evidence for increased DAO in the cerebellum of schizophrenia patients, but the data in other brain regions is still ambiguous. For many years, the cerebellum has been considered as a coordinator of motor function. Nevertheless, the cerebellum is also thought to influence cognition through its connections to the frontal cortex via the thalamus (cortico-cerebellar-thalamic-cortical network) (Koziol et al., 2014). Thus, it has been hypothesised that the structural and functional abnormalities observed in the cerebellum of schizophrenia patients (Bernard and Mittal, 2015; Moberget et al., 2017) disrupt the cortico-cerebellar-thalamic cortical network ultimately leading to cortical dysfunction contributing to the cognitive deficits of schizophrenia (Parker et al., 2014).

An earlier post-mortem study showed an increased *DAOA* mRNA in the prefrontal cortex of schizophrenia brains compared to healthy controls (Korostishevsky et al., 2006), which has not been replicated in the subsequent study (Benzel et al., 2008). Studies have shown increased DAOA protein levels in the plasma of schizophrenia compared to healthy controls (Lin et al., 2014; Akyol et al., 2017), which could not be replicated in a recent study (Ishiwata et al., 2017). Thus, the role of DAOA in schizophrenia remains elusive. Previous post-mortem studies showed decreased NMDA receptor subunits *NR1*, *NR2A*, and *NR2C* mRNA levels (Sokolov, 1998; Beneyto and Meador-Woodruff, 2008; Weickert et al., 2013) and NR1 protein levels (Weickert et al., 2013) in the frontal cortex of schizophrenia patients. However, several studies could not replicate these NMDA receptor subunit mRNA (Akbarian et al., 1996; Le Corre et al., 2000; Dracheva et al., 2001; Rao et al., 2012) and protein findings (Kristiansen et al., 2006, 2010; Rao et al., 2012). In summary, the molecular evidence for the NMDA receptor hypofunction in schizophrenia post-mortem brains remains unclear.

Although the mRNA and protein expression patterns of DAO, DAOA, and NMDA receptor subunits are ambiguous in the frontal cortex of schizophrenia patients, this brain region has been implicated in the pathophysiology of

schizophrenia (Zhou et al., 2015). The prefrontal cortex is involved in executive functions, such as working memory, attention, and decision making, as well as emotional processing, including affection, emotion, and social behaviour (Sakurai et al., 2015). Thus, it has been hypothesised that abnormalities in prefrontal cortex might manifest as cognitive deficits of schizophrenia, and the alleged anatomical and functional dysconnectivity between the prefrontal cortex and other brain regions might contribute to positive and negative symptoms of schizophrenia (Zhou et al., 2015).

***DAO* and *DAOA* gene polymorphisms in schizophrenia**

A landmark study in 2002 identified *DAO* and *DAOA* as putative risk genes for schizophrenia (Chumakov et al., 2002). This study identified 4 *DAO* SNPs (rs211902, rs3918346, rs3741775, rs3918347) and 6 *DAOA* SNPs [rs3916965 (M12), rs3916967 (M14), rs2391191 (M15), rs778293 (M22), rs3918342 (M23), rs1421292 (M24)] to be significantly associated with schizophrenia. This study also found a marginal epistasis between *DAO* rs3741775 and *DAOA* rs778293 SNPs, which has not been replicated in the later studies (Schumacher et al., 2004; Corvin et al., 2007b). Subsequently, many genetic studies have investigated the association of *DAO* and *DAOA* SNPs with schizophrenia, these studies were combined in the SzGene meta-analysis (Allen et al., 2008), which identified 1 *DAO* SNP (rs4623951) and 2 *DAOA* SNPs (rs778293 and rs3916971) to be significantly associated with schizophrenia. In our meta-analysis, we also found the same *DAO* and *DAOA* SNPs to be significantly associated with schizophrenia due to the unavailability of new association studies for these SNPs. In our at-risk for psychosis study, *DAO* and *DAOA* SNPs did not emerge as predictive markers for the transition to schizophrenia-spectrum disorders in at-risk for psychosis population. Furthermore, *DAO* and *DAOA* genes are linked to the glutamate hypothesis of schizophrenia, which is thought to explain the pathophysiology underlying negative symptoms and cognitive deficits of schizophrenia better than the dopamine hypothesis (Frohlich and Van Horn, 2014). Therefore, we integrated genetic information (*DAO* and *DAOA* SNPs) and symptom dimensions (negative symptoms and cognitive deficits), which is one of the core principles of the RDoC framework (Cuthbert, 2014). We found *DAO* and *DAOA* SNPs to be associated with symptoms such as hopelessness (RDoC negative valence system) and perception disturbances (RDoC cognitive system). Thus, these gene polymorphisms may improve our ability to identify at-risk for psychosis who will develop some specific symptoms of schizophrenia. The mechanism underlying genetic association of *DAO* and *DAOA* SNPs with schizophrenia remains unclear. Since the associated *DAO* and *DAOA* SNPs are

in the non-coding region, it is likely that these SNPs exert a pathophysiological effect by modulating DAO and DAOA expression, which still needs to be elucidated (Verrall et al., 2010; Sacchi et al., 2016).

DAO and DAOA interactions leading to NMDA receptor hypofunction

In 2002, Chumakov and colleagues identified a primate specific *DAOA* gene and showed that DAOA increases DAO activity (Chumakov et al., 2002). Following this study, many studies reported contradictory results with a positive effect (Chang et al., 2013), negative effect (Sacchi et al., 2008; Birolo et al., 2016), and no effect (Kvajo et al., 2008) of DAOA on DAO activity. Thus, there is still no consensus regarding the effect of DAOA on DAO activity in the field. To our knowledge, there are no studies to date, which shows the effect of DAOA on NMDA receptor activity *in vitro*. In our study, we wanted to determine the effect of DAOA on DAO and NMDA receptor activity, however, our results were also inconclusive because of the reasons already discussed.

In addition to regulating DAO activity, DAOA has been reported to be localised in the mitochondria and modulate mitochondrial function (Kvajo et al., 2008). A study showed that DAOA binds and impairs the function of mitochondrial methionine-R-sulfoxide reductase B2 (MSRB2), which is responsible for the elimination of cellular reactive oxygen species (ROS), ultimately leading to oxidative stress (Otte et al., 2014). Another recent study also reported a link of DAOA with ROS production (Wang et al., 2015). Interestingly, mitochondrial dysfunctions have been implicated in the pathophysiology of schizophrenia (Park and Park, 2012). Nevertheless, it is still an open question whether DAOA plays a role in the pathophysiology of schizophrenia via NMDA receptor hypofunction or via oxidative stress or both. Thus, unravelling the mystery of DAOA is important with the hope of developing novel pharmacotherapy for schizophrenia targeting DAOA.

Future perspectives

Despite the fact that this thesis revealed the expression trajectory of DAO and DAOA during development and aging in the human brain, the role of *DAO* and *DAOA* SNPs and mRNA as predictive markers for transition to schizophrenia-spectrum disorders and RDoC endophenotypes in at-risk for psychosis population, and the effect of DAOA on DAO and NMDA receptor activity in human cell lines, there are still several unanswered questions, which needs to be addressed in future studies. Future human post-mortem studies should concentrate on identifying the critical regulators (e.g., non-coding RNA) of DAO and DAOA expression across different brain regions and ages to

understand the role of these regulators in the pathophysiology of schizophrenia. The role of *DAO* and *DAOA* genes in neurodevelopment can also be studied using an *in vitro* model of human brain development i.e., human induced pluripotent stem cells (hiPSC) derived three-dimensional organoid culture system called cerebral organoids (Lancaster et al., 2013). Future genetic studies in the field of early recognition and intervention of psychosis should concentrate on increasing the sample size and follow-up period, recruiting more homogeneous at-risk for psychosis individuals and quantifying DAO and DAOA proteins as predictive markers for the transition to schizophrenia-spectrum disorders and RDoC endophenotypes in the plasma of at-risk for psychosis individuals. Studies should also focus on understanding the effect of schizophrenia associated *DAO* and *DAOA* SNPs on their expression so that eventually the role of these SNPs in the pathophysiology of schizophrenia can be elucidated. To understand the effect of DAOA on DAO and NMDA receptor activity, more realistic *in vitro* model i.e., hiPSC derived neurons and glia that recapitulate the complexity of tripartite synapse with the use of gene editing tools, might help in uncovering the mystery of the effect of DAOA on DAO and NMDA receptor activity. Despite years of research in the field of schizophrenia, the pathophysiology of the disorder remains elusive, and the current antipsychotics alleviate mainly the positive symptoms of schizophrenia. Thus, there is an urgent need for novel pharmacotherapy that alleviates negative symptoms and cognitive deficits of schizophrenia. To reach this goal, future studies should focus on elucidating the pathogenesis of schizophrenia with the hope of identifying new therapeutic targets for the treatment of schizophrenia.

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8. List of Abbreviations

1321N1	human astrocytoma cell line
5-HT	5-hydroxytryptamine
AARS	alanyl-tRNA synthetase
ACC	anterior cingulate cortex
ACTB	β-actin
ALAS1	aminolevulinate synthetase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APSS	attenuated positive symptoms syndrome
BAI	beck anxiety inventory
BLIPS	brief limited intermittent psychotic symptoms
cDNA	complementary DNA
CDSS	Calgary depression rating scale for schizophrenia
CHR	clinical high risk for psychosis
CI	confidence interval
CNRQ	calibrated normalized relative quantities
CNS	central nervous system
CNV	copy number variation
CO_2	carbon dioxide
COGDIS	cognitive disturbances
COPER	cognitive-perceptive basic symptoms
CSF	cerebrospinal fluid
DAO/DAAO	D-amino acid oxidase
DAOA/G72	D-amino acid oxidase activator
DNA	deoxyribonucleic acid
DSM-5	diagnostic and statistical manual of mental disorders, 5th edition
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENIGMA	enhancing neuroImaging genetics through meta-analysis
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

gDNA	genomic DNA
GEO	gene expression omnibus
GFP	green florescent protein
GWAS	genome-wide association studies
hDAO	human D-amino acid oxidase
HEK293	human emberonic kidney 293 cell line
hiPSC	human induced pluripotent stem cells
HR	high risk
HWE	hardy-weinberg equilibrium
I^2	inconsistency index
ICD-10	international classification of diseases, 10th revision
kDa	kilodalton
LA	last available follow-up time point
LD	linkage disequilibrium
LG72	long form of G72/DAOA
Ln (OR)	natural logarithm of OR
LSD	lysergic acid diethylamide
MAF	minor allele frequency
MD	molecular dynamics
MD	molecular dynamics
miRNA	microRNA
mRNA	messenger RNA
MSRB2	methionine-R-sulfoxide reductase B2
Na_2HPO_4	sodium phosphate dibasic
NIH	national institutes of health
NMDA	N-methyl-D-aspartate
NR1	NMDA receptor subunit 1
NR1/NR2A HEK293	HEK293 cells stably expressing NMDA receptor subunits NR1 and NR2A
NR2A	NMDA receptor subunit 2A
NRG1	neuregulin 1
NTC	no-template controls
OR	odds ratio
pA	picoampere
PANSS	positive and negative syndrome scale
PBS	phosphate buffered saline
PBST	1x PBS pH 7.4 with 0.1% Tween-20
PCR	polymerase chain reaction
PDB	protein data bank

pF	picofarad
PGC	psychiatric genetics consortium
PLP	pyridoxal phosphate
PME	particle mesh ewald
PME	particle mesh ewald
PPIA	peptidyl prolyl isomerase A
PRISMA	preferred reporting items for systematic reviews and meta-analyses
qRT-PCR	quantitative real-time reverse transcription-PCR
R18S	ribosomal RNA
RDoC	research domain criteria
RNA	ribonucleic acid
ROS	reactive oxygen species
RPL13A	ribosomal protein L13a
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SEM	standard error of mean
SH-SY5Y	human neuroblastoma cell line
SIPS	structured interview for prodromal syndromes
SNP	single nucleotide polymorphism
SPECT	single-photon emission computed tomography
SPI-A	schizophrenia proneness instrument-adult version
SPI-CY	schizophrenia proneness instrument-child and youth version
SRR	serine racemase
SZGene/SzGene	schizophrenia gene [web-based schizophrenia meta-analysis database; http://www.szgene.org/]
UHR	ultra-high risk
XPNPEP1	X-prolyl aminopeptidase1
ZInEP	Zurich program for sustainable development of mental health services

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10. Curriculum Vitae

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EDUCATION

Sept 2014 - present	**Ph.D. Neuroscience** under the supervision of Prof. Dr. Edna Grünblatt, Molecular and Neurobiochemistry laboratory, Department of Child and Adolescent Psychiatry and Psychotherapy, University of Zurich, Switzerland as part of Zentrum für Neurowissenschaften Zürich (ZNZ) International Ph.D. Program Topic: D-amino acid oxidase activator (DAOA/G72) pathways and its role in Schizophrenia
Oct 2012 - Sept 2014	**MSc Integrative Neuroscience** from Otto-von-Guericke University (OvGU), Magdeburg. Master thesis under the supervision of Prof. Dr. Daniela Dieterich and Dr. Peter Landgraf, Institute of Pharmacology and Toxicology, Otto-von-Guericke University, Magdeburg, Germany. Topic: Assessment of protein turnover rates in primary cortical cultures
Mar 2012	Registered with the Karnataka Medical Council, India. Registration No.: 93815
Aug 2006 - Feb 2012	**Bachelor of Medicine and Bachelor of Surgery (MBBS)** from Kasturba Medical College (KMC) Mangalore, Manipal University, India
June 2004 - May 2006	Pre-University Course (PUC) from MES College, Malleswaram, Bangalore, India
June 1994 - May 2004	First to Tenth Standard from St. Anne's School, Maddur, Mandya District, India

AWARDS

Sept 2014 - Sept 2017	Swiss Government Excellence Scholarship 2014-2017 (2014.0826)
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CONFERENCES AND POSTER PRESENTATIONS

8-10 Nov 2017	ECNP Workshop on Clinical Research Methods at Barcelona, Spain
9-12 Mar 2017	ECNP Workshop on Neuropsychopharmacology at Nice, France, presented a poster
2-6 July 2016	10^{th} FENS Forum of Neuroscience at Copenhagen, Denmark, presented a poster
24 Jan 2015 23 Jan 2016 27 Jan 2017	Swiss Society for Neuroscience (SSN) annual meeting at the University of Fribourg, University of Lausanne, University of Basel, Switzerland, presented a poster
22 Oct 2014 17 Nov 2015 25 Oct 2016	1^{st}, 2^{nd} and 3^{rd} Burghölzli Psychiatry Meeting, Zurich, presented a poster
11 Sept 2015 15 Sept 2016 14 Sept 2017	ZNZ Symposium 2015, 2016 and 2017, Zurich, Switzerland, presented a poster
09 Jun 2015	Psychiatric research groups scientific exchange, Schlieren, Switzerland, oral presentation

PUBLICATIONS

Jan 2018	**Jagannath V**, Gerstenberg M, Correll CU, Walitza S, Grünblatt E (2017). A systematic meta-analysis of the association of Neuregulin 1 (*NRG1*), D-amino acid oxidase (*DAO*) and DAO activator (*DAOA*)/*G72* polymorphisms with schizophrenia. J Neural Transm (Vienna). 125(1):89-102. doi: 10.1007/s00702-017-1782-z

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11. Declaration

I, Vinita Jagannath hereby declare that this is my original work. The data analysis, figures, tables, and writing in this thesis was primarily performed by me. I have duly acknowledged all source of information, which was used in this thesis. This thesis has not been submitted for any degree in any other university. The work for 4 paper sections enclosed in this thesis was done by me, with normal supervisorial input from Prof. Dr. Edna Grünblatt. My contribution to each paper section is as follows:

	Title of the manuscript	**Contribution of Vinita Jagannath**
1	**Expression of D-amino acid oxidase (*DAO*/*DAAO*) and D-amino acid oxidase activator (*DAOA*/*G72*) during development and aging in the human post-mortem brain**. Published in Frontiers in Neuroanatomy, April 2017, 11:31	Vinita Jagannath performed the experiments, analysed the data, and wrote the paper
2	**A systematic meta-analysis of the association of Neuregulin 1 (*NRG1*), D-amino acid oxidase (*DAO*), and DAO activator (*DAOA*)/*G72* polymorphisms with schizophrenia**. Published in Journal of Neural transmission, January 2018, 125(1):89-102	Vinita Jagannath performed the literature research, analysed the data, and wrote the paper
3	**Prediction analysis for transition to schizophrenia in individuals at clinical high-risk for psychosis: The relationship of *DAO*, *DAOA* and *NRG1* variants with negative symptoms and cognitive deficits**. Published in Frontiers in Psychiatry, December 2017, 8:292	Vinita Jagannath performed the experiments, analysed the data, and wrote the paper
4	**Controversial effects of D-amino acid oxidase activator (DAOA)/G72 on D-amino acid oxidase (DAO) activity in human neuronal, astrocyte, and kidney cell lines: The N-methyl D-aspartate (NMDA) receptor hypofunction point of view**. Published in Frontiers in Molecular Neuroscience, October 2017, 10:342	Vinita Jagannath designed and performed the experiments, analysed the *in vitro* data, and wrote the paper

Place, Date: Zurich, 05.02.2018 Vinita Jagannath